Detection, quantification, and concentration of *Pf*HRPII in urine of malaria patients: implications for urine-based *Plasmodium falciparum* diagnostics.

by
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Abstract

Plasmodium falciparum remains a major source of global mortality, causing nearly 500,000 deaths per year. Although diagnostics have made major improvements in recent years, microscopy on blood smears remains the gold standard, with blood-based rapid diagnostic tests (RDTs) as the other major player in field tests. However, there remain social challenges as well as health concerns associated with taking blood, and discovery of an alternate body fluid diagnostic has been an area of major research. Previous attempts at urine-based diagnostics have generally suffered from impaired sensitivity, particularly among afebrile patients. This is likely to be due to a decreased quantity of the antigen being detected (*Pf*HRPII) in urine as compared to plasma. This work uses urine and plasma samples collected from patients at a health post in Kpone on Sea, Ghana, of which 64% were malaria-positive by microscopy, to examine this dilution factor and other correlates of urine *Pf*HRPII detection. The first aim was to detect *Pf*HRPII in the urine using Western Blot, dot blot, and ImmunoPCR, and compare these to a buffered urine-specific RDT. Of these, ImmunoPCR was the most sensitive, and in each subset there was a higher proportion of positive results in febrile patients. The next aim was to quantitatively examine the ratio of *Pf*HRPII in plasma versus urine using both ELISA and ImmunoPCR techniques, which yielded a geometric mean ratio of 69.0 and 137.6 respectively. The implication for urine-based diagnostics is that they will require an almost 100-fold (or more) increase in sensitivity or sample volume to be viable and comparable to the sensitivity of blood-based diagnostics. Thus, our final aim was to attempt to rectify this by applying Nanotrap nanoparticle technology to sequester and concentrate *Pf*HRPII in 500 microliter aliquots of urine. Although the nanoparticles were able to concentrate the protein five or six fold, this was insufficient to counteract the dilution from plasma and was much below the theoretical 31-fold increase in protein content corresponding to a 31-fold increase in sample volume. Further, this technology

was suboptimal for detection in actual patient samples, showing concentration in only one sample. Although there is room for increasing sample size and altering composition of nanoparticles, to surmount the obstacle of decreased *Pf*HRPII concentration in urine. Additionally, due to the high variability in *Pf*HRPII detection in the urine, any adequate diagnostic will likely have to go well above and beyond the 100-fold benchmark elucidated in this work in order to capture a meaningful proportion of malaria-infected patients.

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Introduction

Infection with human *Plasmodium* is an important source of global mortality and morbidity, estimated to infect over 200 million people[1]. Malaria the disease is caused by a discrete group of apicomplexan parasites belonging to the genus *Plasmodium* that are transmitted through the bites of the female *Anopheles* mosquito. There are five major species that have been shown to infect humans; *P. malariae, P. ovale, P. vivax, P. knowlesi,* and *P. falciparum*. Each has a slightly different global distribution, but all are located in the tropical belt in which the vectors make their home. Of these, the most lethal is *P. falciparum*, which is mostly concentrated in the African region, although a significant number of infections also occur in Southeast Asia[2]. Although malaria, which was introduced with the slave trade, was found throughout the United States, it was effectively eliminated in 1951[3]; in modern times, US infections are almost always brought back by travelers, rather than being caused by local transmission. The 2013 Malaria Surveillance Report by the CDC found 1,727 cases of malaria (mostly imported) were reported during 2013[4]. This effective elimination was the result of a wide array of public health interventions in the first half of the 20th century, and has been aided by overall economic development in the US. Nowadays, malaria is largely a disease found in lower-income countries due to lack of appropriate infrastructure and preventative measures, and continues to be one of the leading causes of death worldwide, particularly in children under 5. In 2015, it is estimated that 438,000 deaths were attributable to malaria infection, of which 90% were in sub-Saharan Africa[1].

Reducing the global burden of malaria-induced disease (and eventually eradicating it) has been a longtime goal of numerous global health organizations, and involves a multipronged approach to block transmission, prevent infection and treatment. On a global level, some measure of success has certainly been achieved; in 1900,

around 77% of the global population was considered malaria-exposed, whereas in 2010 only about 50% was exposed[5]. One of the cornerstones of any disease control scheme, both in terms of individual treatment as well as community-level surveillance, is an appropriately specific and sensitive diagnostic method – without one, it is impossible to track effectiveness of interventions or appropriately target treatment. The majority of malaria diagnostics are blood-based, which has been met with resistance in many countries for community screening of healthy individuals. The goal of this work is to test the hypothesis that urine *Pf*HRPII can be used as a biomarker for clinical or subclinical infection. This paper seeks to identify and quantify P*f*HRPII in plasma and urine to compare their abundance and explore novel technologies for concentration of proteins in urine, with the long-term goal of contributing valuable information to the burgeoning field of urine-based diagnostics.

Malaria Transmission Cycle and Control

The complex life cycle of the *Plasmodium* parasite allows for multiple potential checkpoints for control. As mentioned, malaria is transmitted by the female *Anopheles* mosquito, which is considered its definitive host (i.e. the host in which it undergoes sexual reproduction). When a female *Anopheles* mosquito feeds on a human host harboring the sexual stages of the malaria – the male and female gametocytes - in the in the blood, the gametocytes undergo a process of sexual reproduction, proliferation, maturation and development within the mosquito that results in the generation of thousands of the infective stage known as the sporozoite. Sporozoites migrate to the salivary glands, where they may be transmitted with the next bite. In the human host, these sporozoites first migrate to the liver, where they begin division and create meronts. Rupturing of meronts releases thousands of merozoites, which invade red blood cells to begin the erythrocytic portion of the life cycle, during which malaria symptoms occur. Merozoites that invade the RBCs may either form erythrocytic schizonts, generating

further merozoites, or differentiate into male and female gametocytes to begin the transmission cycle anew with the bite of another *Anopheles* mosquito[6,7]. Figure 1 shows a schematic representation of this complex lifecycle.

The importance of the mosquito vector to the maturation and transmission of *Plasmodium* parasites makes them an attractive intervention point for reducing burden of disease, and many of the most important contributions to reducing malarial disease have come from vector control strategies. Because *Anopheles* mosquitoes tend to bite at night, insecticide-treated nets (ITN) have been found to be effective, reducing parasite prevalence by up to ten-fold in one study[8] . ITNs work by inhibiting bites through physical protection by the mesh of the net as well as harboring pyrethroid insecticides within the fabric to repel and kill the mosquitoes entirely[9]. Indoor residual spraying (IRS) is another intervention targeted at reducing mosquito bites and transmission by coating the interior of homes with insecticides that kill *Anopheles* mosquitoes. IRS tends to have more community-wide benefits in areas of high coverage, even amongst those whose houses were not sprayed, whereas net usage is largely protective only of the particular people using the nets[10]**.** Both have their drawbacks; nets are sometimes either unintentionally misused or purposely utilized for other purposes, and both require some level of upkeep. However, they have both contributed greatly to malaria control efforts worldwide. One study found ITNs in particular to be the greatest contributor to decreasing the number of cases of malaria in Africa between 2010 and 2015[11]. Other studies have shown an additive protective effect ITN and IRS in areas where both ITN and IRS interventions have been deployed[12].

On the human side of the transmission cycle, mass drug administration (MDA) or taking antimalarials prophylactically are also options. Drugs used for mass administration include primaquine, pyrimethamine, and chloroquine, among others[13]. Concerns about exacerbating tendencies towards drug resistance in parasites have

tempered the use of MDA, but recently MDA has begun to be seen as a potential tool in areas approaching eradication. Drawbacks include treatment of women of childbearing age by exposing them to unnecessary drugs and treatment of more than 99% of population which may be not infected. Currently, the WHO recommends it only be used in "ideal" conditions where compliance will be high and elimination is feasible and close at hand[14], as its effectiveness in more high-transit areas and past six months post-treatment is uncertain[15]. Some mathematical models imply that MDA could be used in concert with other interventions to drastically reduce transmission[16]. Regular prophylactic consumption of anti-malarials such as chloroquine and mefloquine[17] is also common, though generally too costly to benefit anyone other than travelers spending short, discrete periods of time in endemic areas.

Malaria Symptoms and Treatment

The most prominent symptom of malaria is fever, which in *P. falciparum*-infected patients typically presents 10-15 days after an infectious bite[1]. Other symptoms include headache, joint pain, anemia, vomiting, myalgia, jaundice, diarrhea, and many others[18,19]. Unfortunately, these can be commonly confused with a number of other illnesses such as pneumonia in children[20,21]. *P. falciparum* is the deadliest of the species partly because it causes a much faster spike in parasitemia, and partly because of its ability to cause cytoadherence in infected erythrocytes[22]. *P. falciparum* is also the only malaria species that cytoadheres to the vascular endothelium in the brain, blocking local circulation and causing the condition known as "cerebral malaria", a severe and complicated set of clinical manifestations (including delirium and coma) that can cause death and lifelong cognitive defects in survivors[23]**.** Cerebral malaria is most prevalent in children under 5, and even with treatment causes death in 15-20% of cases (without treatment, death is virtually inevitable)[23].

Treatment of malaria is a constantly evolving field, partly driven by drug resistance, drug availability and cost. Traditional therapies such as chloroquine and sulphadoxine-pyrimethamine cost only a few cents per dose, but rampant resistance has rendered them ineffective[24]. Current approaches center on artemisinin combination therapies (ACT), which cost $2.00-$2.50 for an adult dose. Although this seems a relatively low price to pay, it represents a large proportion of income for most in the poverty-stricken countries where malaria is endemic. In addition, inaccurate assessment of disease and provision of unnecessary drugs can also cause significant strain on already thinly-stretched health resources in places where these drugs are partly or wholly subsidized. This can lead to stock-outs, which may occur in as many as 84% of public outlets (as an example from Kenya)[25] and may also result in the creation of private, for-profit drug vendors that further increase prices, or create opportunities for the introduction of counterfeit drugs. Additionally, continued development of resistance has created the need for even more costly combinations of drugs. The cost-effective drug of choice for any particular area varies depending on local transmission; for example, it was found in Tanzania that investing in more costly ACT combinations prevented treatment failure, but in other areas this may represent an unnecessary expense[26].

The challenge in efficient prescription of antimalarials is that symptoms of malaria are frequently nonspecific, and clinical presentation may be confused with a variety of other diseases. Even a sophisticated clinical algorithm may result in the prescription of antimalarials to ~29% of non-malarial cases and neglect up to half of actually malaria-infected persons[27]. Another study found that only ~10% of children presenting as febrile actually had malaria[28]. At some health posts, fever is considered enough of a reason to prescribe antimalarials even without any confirmatory parasitological diagnosis, making this statistic particularly alarming. A study in Uganda determined that while 73% of febrile patients received antimalarials, only 35% had positive rapid diagnostic test (RDT)

results, and overall appropriate treatment was only 34%[29]. Economic studies have implied that ready access to subsidized, over-the-counter antimalarials vastly increases coverage, but that nearly half of such pills wind up in the hands of patients without malaria; the overall recommendation was for an additional subsidy for over-the-counter diagnostics as an addition to malaria control regimes[30]. Provision of antimalarials to those without malaria is not solely a waste of money; it also increases the likelihood of development of resistance in parasites and, when using sulfa drugs, bacteria. . Another model showed that the cost-per-life-saved of ACT treatment for children under five years of age was $209 with presumptive treatment (based only on fever) and $171 with an RDT confirmation of malaria infection[31]; with several million malaria infections occurring in children each year[32], this cost differential adds up . Clearly, misdiagnosis and inaccurate provision of antimalarials present fiscal challenges in addition to potentially perpetuating the advent of resistant strains of parasites with the widespread circulation of antimalarials. Thus, having field-implementable, accurate diagnostics is a critically important piece for efficient treatment and effective global malaria eradication.

Malaria Diagnostics

Traditionally, malaria diagnostics have centered on the use of microscopy to identify parasites in blood films during a clinical illness. Although this is still considered the “gold standard” in most areas, it is far from a perfect solution. Although microscopy has the potential to detect quantities of *P. falciparum* as low as 50 parasites per microliter of blood[33], in practice the limit of detection may be higher than 500 parasites per microliter of blood[34] due to inadequate training, patience or expertise of microscopists. Aside from issues with sensitivity, improper use and inadequate training may also lead to inflated numbers and over-detection of the parasite. One study demonstrated a five-fold over-diagnosis of *P. falciparum* infection using microscopic

techniques compared to an RDT (though this does not address the possibility that the RDT was simply not detecting low-level infections)[35]. Microscopy requires resources that are frequently unavailable in the low-resource settings where malaria is endemic, and cutting corners for field-adaptable technology can cause specificity to suffer. Field microscopy may have a positive predictive value as low as 43.2% as compared to "expert" microscopy done with better equipment, in part due to more rudimentary field technology[36]. Quality of training may also be an issue. This limits the efficacy of microscopy as a field-implementable test for malaria, and contributes to inaccurate (or nonexistent) confirmatory diagnosis in clinical settings. This, as mentioned, is problematic due to both extra costs associated with unnecessary treatment as well as the burgeoning effects of resistance to anti-malarial drugs, which is exacerbated by over-prescription.

The "Rapid Diagnostic Test", or RDT, is the most prevalent modern-day "rival" to microscopy in terms of field-based malaria tests. RDTs function on fairly basic principles: the presence of the parasite antigen (usually histidine-rich protein 2, *Pf*HRP2) in the blood sample causes an antibody-based reaction that ultimately creates a band on the nitrocellulose strip of the diagnostic implement. These fall under the umbrella of "lateral flow assays"; other examples include the common pregnancy test or urine *Streptococcus pneumoniae* test. Malaria rapid tests may also include antibodies for aldolase and lactate dehydrogenase, allowing for some level of speciation, although mixed infections still present problems[33]. The sensitivity of the best RDTs is 75-99% as compared to microscopy, depending on local transmission levels and other factors (such as parasitemia)[37]. These are theoretically easy to use and deploy in a field setting, do not require electricity, and are thus more widely usable than microscopy. However, they do not provide information on the level of parasitemia and severity of infection, and are unable to distinguish mixed infections with multiple species of *Plasmodium*. There have

also been studies citing widespread misinterpretation of bands, including a lack of attention to the control band or failure to recognize weak positive bands[38].

Laboratory-based diagnostics such as PCR have thus far been shown to be the most sensitive method of detecting parasite in the blood. Although there are a wide variety of potential molecular targets, the most commonly used is an 18S ribosomal RNA sequence; this has the additional benefit of having multiple (~3) copies per parasite, allowing for increased sensitivity over other potential targets. Nested PCR, quantitative PCR, and nucleic acid sequence-based amplification are all molecular techniques currently in use[39]**.** qRT-PCR was found to have an analytical sensitivity of $\geq$ 20 parasites/mL, with few false positives and a propensity for detecting infection 3.7 days earlier as compared to blood smears[40]. Currently, some scientists are pushing for increased use of these molecular diagnostics in epidemiological studies as a method of detecting submicroscopic and asymptomatic reservoirs of parasite that may be contributing significantly to transmission, particularly in low-transmission areas that are nearing elimination[39,41]. However, none of these techniques are easily field-implementable, as they require refrigerated items and electricity to be run.

Enzyme-linked Immunosorbent Assays (ELISAs) are also considered a validated malaria diagnostic[42], although they face this common pitfall of requiring lab equipment (i.e. a plate reader). Most ELISAs detect the presence of parasite proteins such as histidine-rich protein 2 (known as *Pf*HRP2, to be discussed later), which is secreted by the *P. falciparum* parasite during all stages. *Pf*HRP2 is specific (of the human malarias) to *P. falciparum*, though some versions of the diagnostic target proteins such as aldolase and lactate dehydrogenase that occur in other species as well**,** allowing for speciation. CelLabs[43] manufactures a specific *Pf*HRP2 ELISA kit that may be used for diagnostic purposes, with an approximate limit of detection of about 100-1,000 picograms of *Pf*HRP2[42,44,45]. Although in some comparative studies this has been shown to outperform

microscopy[46] and near the performance of qPCR, other studies have placed the overall sensitivity at about that of a rapid diagnostic test, or RDT[44]. Tests for antibodies to malaria may also be done, though they are uncommon in the context of diagnosing clinical malaria due to the amount of time it takes to mount an antibody response.

Additionally, there has long been a desire to develop a diagnostic utilizing fluids other than blood. There tends to be more resistance to collection of blood samples, and in the case of co-infections with other diseases (such as HIV), exposure to blood on the part of field staff may present risk of exposure and transmission, especially with inadequate gloves and protective equipment. One common negative association and source of resistance to the RDT is that blood tests are equated with HIV tests, which carry a great amount of stigma[47]. Other sources cite local religious beliefs that blood is sacred as major social factors for eschewing the RDT[38]. Although the use of the finger sticks has become fairly widespread and has gained traction in communities, finding an alternative option would be ideal.

Past attempts to use other, more easily collected, bodily fluids (such as urine) have met with limited success. It has long been known that proteins associated with *Plasmodium* infection can be found in the urine; rudimentary studies as early as 1991 have detected up to 19 different malarial proteins in the urine[48]. In 2004, the first attempts to use urine for malarial diagnosis attempted to apply urine directly to an RDT designed for blood (in this case, the ParaSight-F test); it suffered from low sensitivity (81%) and very low specificity (26%)[49]. One of the problems with this application was that the low pH of urine interferes with antibody-antigen binding, thus hampering the functioning of the RDT. In addition, the concentration of these malarial proteins is likely to be much lower in the urine than in the bloodstream. Overall protein content of plasma is generally 400 times higher than that of urine[50]. This presents significant challenges for maintaining sensitivity of any diagnostic implement, as even with high levels of blood

*Pf*HRP2 we may see moderate or low levels of *Pf*HRP2 in urine. The Urine Dipstick test designed by Fyodor, for example, also operates on the basis of detection of *Pf*HRP2, and when evaluated in 2014 had only moderate sensitivity compared with a blood smear (although it did address the pH issue by incorporating a buffer into the test). Overall sensitivity was found to be 83.75%, but for infections with a parasite density less than 200 parasite/microliter, this dropped to 50%[51]. This is problematic, because any adequate malaria elimination strategy will need to find all potential sources of parasite in the population (i.e. even those with lower parasitemia) in order to eliminate potential reservoirs. Even at an individual clinical level, the WHO threshold for adequate malaria diagnostics is 75% sensitivity for 200 parasites per microliter, and the current Dipstick test fails to make the cut[52].

HRP2

Most of our modern diagnostics focus on finding *Pf*HRP2 in assorted body liquids. As such, it is important to understand *Pf*HRP2 as a protein. PfHRP2 is, as the name suggests, abundant in histidine as well as alanine with numerous repetitive epitopes and B-cell epitopes that allow for easy antigen capture using antibody-based techniques. It is about 29 kilodaltons in size, but frequently runs as a dimer on SDS gels. It is considered a good candidate for a biomarker of malarial infection because it is present in the blood regardless of the location of the parasite, and is synthesized and excreted by the parasite in multiple of its lifecycle stages in the human host, although most is released in schizont rupture[53,54]. This also means that it may be more representative of total parasite biomass, including sequestered parasites, as secretion is not limited to circulating parasites[55].

*Pf*HRP2 also has a long half-life, which is both a blessing and a curse. On the one hand, it is relatively sturdy and does not degrade easily, because of rare protease

sites and easier to find in the urine (and in samples that may have been freeze-thawed repeatedly). Indeed the ParaSight F test was used to diagnose malaria in mummies. However, its persistence in the blood stream also makes post-treatment diagnosis difficult; even if the parasites themselves have all been eliminated, *Pf*HRP2 may linger for multiple weeks, creating false positives (or making it impossible to determine whether or not all parasites were truly killed)[33,56].

There is also some controversy over correlating levels of *Pf*HRP2 with parasitemia. One study examined different parasite isolates and found a wide range of secreted protein levels even when controlling for parasitemia, finding little correlation between initial blood parasitemia and protein concentrations for Colombian isolates[57]. This, the authors claimed, ran counter to previous data on isolates from Thailand which found high correlation between parasitemia and *Pf*HRP2 levels[54,55]. This may be partly because the Colombian study relied on parasitemia gleaned from blood films and may not have taken into account the sequestered biomass, as was done in the studies using Thai samples. Thus far, the general consensus is that *Pf*HRP2 is unlikely to be well-correlated with circulating parasitemia[58], though this may be points in its favor as it more accurately represents the sequestered biomass which tends to cause clinical complications.

In addition, a number of studies have examined the clinical utility of quantified *Pf*HRP2 determination in predicting clinical outcomes. High levels of *Pf*HRP2 have been associate with cerebral malaria (possibly via heightened *Pf*HRP2 levels in cerebrospinal fluid)[59], severity of malarial illness in adults[55] and severity of malaria, depth of coma, and mortality in children[58].

The future of malaria diagnostics

Malaria continues to be a hot topic in global health circles, both within the laboratory-based community and outside. There is significant pressure to increase the sensitivity of existing diagnostics in order to more accurately discover asymptomatic and submicroscopic carriers of the parasite (which may continue to impede total eradication) as well as devise novel mechanisms to test alternative body fluids. In an ideal world, a malaria diagnostic that did not involve a finger stick would be available that could be done and accurately interpreted in the home, by the patients themselves.

As evidenced by the inadequacy of previous attempts at urine-based diagnostics, there is some level of dilution and potentially degradation that occurs during the processing of blood. A major question that still needs to be answered, however, is this: How much more dilute can we expect urine samples to be? Although there is data for overall protein content of blood versus urine, it is unclear whether this will directly translate for *Pf*HRP2, particularly given potential increases in protein shedding during severe malarial episodes. In order to understand the "hurdle" that must be jumped, it is important to have a quantitative notion of how much more sensitive tests must be to be viable in urine. To this end, this study utilizes both ImmunoPCR techniques (previously used to examine *Pf*HRP2 in cerebrospinal fluid[59]) and a *Pf*HRP2-specific ELISA kit[43] to compare matched samples of plasma and urine from febrile patients in Ghana.

The question then becomes how we might specifically concentrate *Pf*HRP2 from body fluids to enable greater sensitivity. Collaborators at George Mason University have previously demonstrated viable "Nanotrap" technology to address this problem The Nanotrap technology is a buoyant, porous "smart" nanoparticle containing a novel high-affinity chemical bait that will rapidly harvest solution-phase analytes, protect the captured molecules from degradation, and exclude high abundance unwanted large proteins, within minutes, in one step[60,61]. The porosity of the nanoparticles can be rapidly

switched under physiologic conditions. The chemical baits, immobilized in the core of the nanoparticles are a previously unexplored class of modified organic molecule dyes that bind molecules with extremely high affinity (KD <= 10-13 M) and a very low off-rate[62]. Upon contact with the biofluid sample, the suspended nanoparticles immediately affinity-sequester desired biomarkers away from albumin, fully protect the biomarkers from degradation during processing (even at elevated temperatures), and massively concentrate the sequestered biomarkers. The technology can dramatically (demonstrated up to 10,000 fold) improve the lower limits of detection and the precision quantification by any analytical method[61,63,64]. To date, successful diagnostics have been made for both Lyme disease[65] and Chagas disease[66] using this technology. We sought to optimize the Nanotrap particles for the concentration of *Pf*HRPII and test their efficacy with both spike-ins of recombinant protein as well as patient samples.

Hypothesis: Urine *Pf*HRPII can be optimized as a biomarker for malaria detection.

Goal: Characterize the amount of *Pf*HRPII in a group of Ghanaian urine samples in order to improve urine malaria detection.

Aim 1: Detect urine *Pf*HRPII by Western Blot and dot blot to compare to previous testing.

Aim 2:Determine the ratio of *Pf*HRPII protein present in plasma versus urine of infected patients using ELISA and ImmunoPCR.

Aim 3: Select and test nanoparticle technology for concentration of *Pf*HRPII in urine samples.

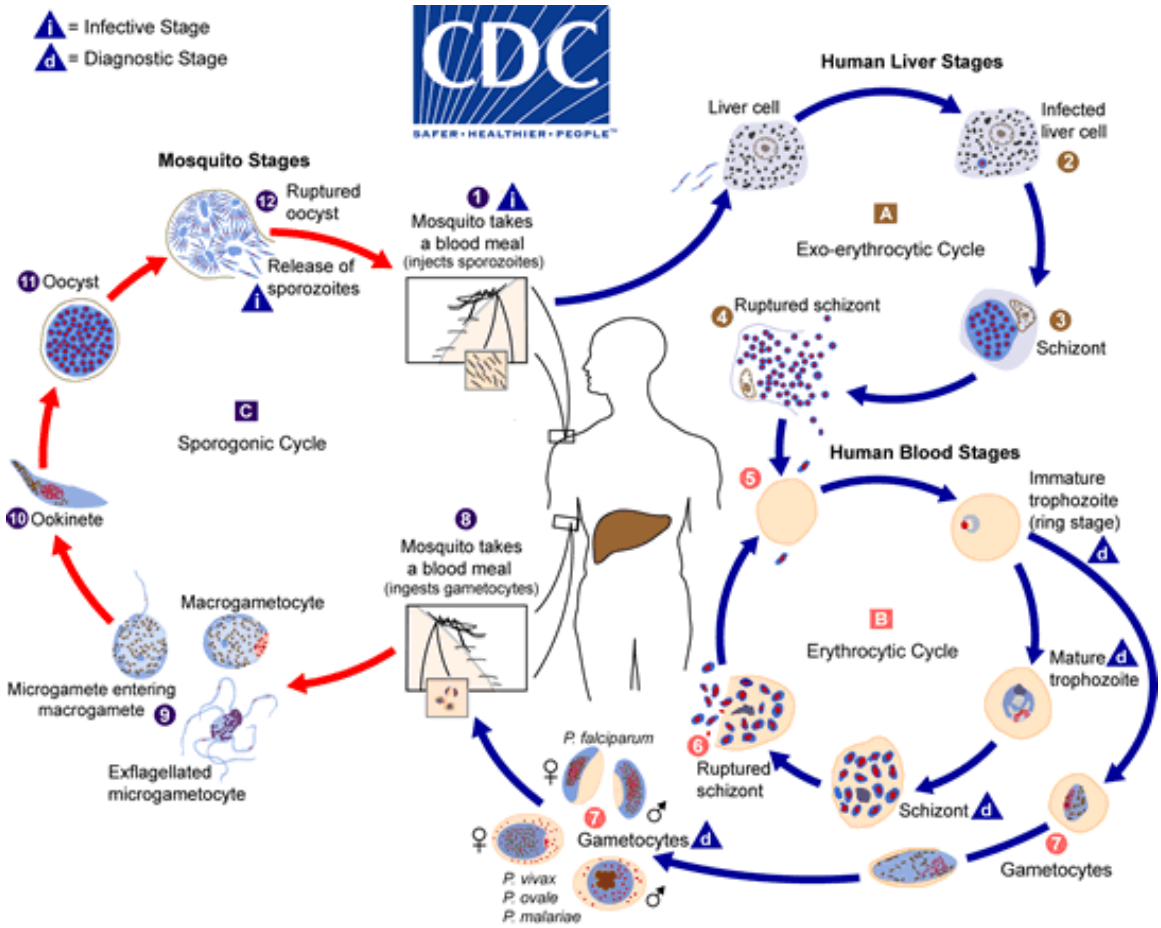


Figure 1. Schematic of malaria life cycle[7].

Materials and Methods

Study subjects

Samples were all obtained from Kpone-on-Sea, a small fishing village on the southeastern coast of Ghana in 2002. The community has been developed as a model site for malaria intervention studies by the WHO-funded Kpone Malaria Project. Peak malarial transmission in this area occurs from May to July and in November with a peak parasite rate of 21% in children under five. At the time of collection the overall prevalence of malaria was about 11%, of which greater than 95% were *P. falciparum.* 290 study subjects were recruited from symptomatic patients at the Kpone Health Center, of which 187 were adults, 58 were pregnant women, and 103 were children. Subjects were deemed inadmissible in the study if they had received a transfusion, had cerebral malaria, or had hemoglobin < 50 g/L.

Information collected from participants included age and axillary temperature. Blood and urine samples were collected for each patient and transported to School of Public Health (SPH) and the Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, Legon, for further processing. Blood samples were used to make thin-film slides (to determine parasite species), thick-film slides (for parasitemia), and for use on a rapid diagnostic test. 20-150 mL of fresh clean-catch urine was collected for each participant, and 202 urine samples were ultimately available for use. Aliquots of samples were sent to Johns Hopkins School of Public Health. Urine samples were adjusted to pH 7-9 and subjected to Biorad CBB protein (Hercules, CA) assay for protein concentration. Urine samples were also tested for presence of *Pf*HRPII using Western and dot blots in 2005, as well as given the Binax buffered urine-specific (BUS RDT) malaria test using 100 microliters of sample for each test). Samples were frozen and stored until the present

analysis in 2016. All plasma and blood samples were diluted 1:100 with sterile 1X PBS for ELISA analysis, and diluted by 1:10 for ImmunoPCR analysis.

Binax buffered urine specific RDT

In 2005 , Binax created a pilot prototype urine malaria test stick utilizing the same aldolase and HRP2 antibodies for the FDA approved Binax Now malaria blood RDT. The test had a large absorbant pad distal to the read zone, wick, conjugate pad and sample pad, Approximately 150 to 200 microL of urine was absorbed up the dipstick to be analyzed. The test has a 40 picogram limit of detection for recombinant HRP2.

Western Blots

Western Blots of samples were initially performed at Johns Hopkins School of Public Health. Those used for nanoparticle analysis were performed at George Mason University using slightly different materials; these are described below with other portions of the nanoparticle protocol.

For initial analysis of samples, 10 microliters of each urine sample was mixed with 3.3 microliters of 4x Sample Buffer (Bio-Rad) and boiled for 10 minutes 95°C. In subsequent blots, different quantities of sample were used to manipulate relative intensities for easier viewing. After boiling, samples were loaded into a 4–20% Mini-Protean TGX Gel (Bio-Rad) for Western Blotting for electrophoresis. After this, proteins were transferred to a nitrocellulose membrane for one hour at 0.400 Amps and 250 V in transfer buffer (made with 3.03 grams Tris-HCl, 14.4 g glycine, 200 mL methanol and 1 mL 10% SDS brought to 1 liter of solution with deionized water**)**. The membrane was then placed in 40 mL blocking solution (5% powdered milk, 2% bovine serum albumin in 0.1% PBS-Tween, diluted by 1:5 with 0.1% PBS-Tween) for one hour at RT. 20 uL of 3A4 monoclonal primary antibody was then added to 40 mL of 1:5 blocking solution. Blots were then incubated in this solution at RT for 90 minutes. After rinsing with 0.1% PBS-Tween five times with five minutes in between each, the membranes were placed in 40

mL of 1:5 blocking solution with 20 uL of secondary goat antimouse IgG + IgM antibody. The membrane was exposed on Amersham Hyperfilm ECL (GE Healthcare, UK) using ECL Western Blot detection reagents (GE Healthcare, Buckinghamshire, UK), using 10 minute exposures.

Coomassie Brilliant Blue

Coomassie Briliant Blue staining was performed on one gel of matched plasma-urine pairs to visually detect other proteins. The gel was run as described for Western Blots, then transferred to a large plastic petri dish and coated in approximately 40 mL of Coomassie Brilliant Blue solution (1g Coomassie Brilliant Blue stain, BioRad, in 1 liter of 40% Methanol, 10% acetic acid, and 30% H2O). This was incubated overnight, then destained repeatedly in 15 minute intervals until the background became clear. Destaining solution was made of 50% H2O, 40% methanol, and 10% acetic acid.

Dot Blots

10 microliters of each sample was mixed with 90 microliters of 1x PBS in separate wells of a 96-well plate. Each of these was then transferred to a 0.45 mm nitrocellulose membrane (Bio-Rad) using a suction-based dot blot apparatus. The membrane was then subjected to the same blocking and antibody steps as membranes from a Western Blot, described above.

Immuno PCR

ImmunoPCR was performed by Dr. Kei Mikita in 2005, as described in his paper quantifying *Pf*HRPII in cerebrospinal fluid[59]. All plasma samples were diluted 1:10, and all urine samples were used without dilution. Briefly, monoclonal antibody 3A4 was used as the capture antibody and incubated overnight at 4 degrees in 96 well microplates. After washing and blocking, 5 uLs of sample were diluted in 15 uL of blocking buffer and

incubated in wells overnight at RT. After a second set of washes, oligo-HRP2 antibody was added and incubated for 2 hours at RT. After washing the oligo-antibody, PCR-grade water was added, followed by sealing the microplates and placing them in boiling water to denature the antigen-antibody-oligonucleotide product. Real-time PCR was then performed to quantify the presence of the oligonucleotide using forward primer (5'-GAC AGC GTA CGA CCA ACC T-3'), reverse primer (5'-GAC CTT GCT GCT GGT ATT TC-3'), Texas red probe (5'-CCG GGT CTG ATC GGC GAT-3') and 4.5 microliters iQ™ Multiplex Powermix (Bio-Rad, USA).

ELISA

A CELISA kit for Malaria Antigen made by CelLabs in Australia was used according to kit instructions[43]. Both plasma and urine samples were diluted with sterile PBS based on preliminary dot blots and ELISAs to bring quantities into the approximate linear range of the test. Previous work in our lab yielded a much smaller range of linearity for the concentration of PfHRP2 than the literature (which found a range of 0.391 to 25 nanograms per 100 uL test sample)[42], ranging from 1 ng down to 0.05 in a 100 microliter aliquot. These concentrations were used as the standard curve for further ELISAs as well as preliminary dot blots. Dilutions ranged from 1:40 to no dilution, and duplicates of each sample were run. Diluted samples were incubated in the wells of the ELISA plate (pre-coated with monoclonal antibodies against *Pf*HRPII) for one hour, then washed five times with wash buffer (PBS-Tween, from kit, diluted 1:20 as described). Subsequently, 100 microliters of freshly diluted conjugate-antibody solution was added to each well for a second one-hour incubation at RT. Following another five washes, 100 microliters of freshly-diluted chromogen substrate was added to each well using a multichannel pipet. The plate was then wrapped in foil and put into a drawer to incubate for 15 minutes

before adding 50 microliters of stop solution. Plates were then read using a spectrophotometer (POLARStar Omega).

For analysis, the background reading for negative controls was subtracted from the raw reading. Samples with a low "adjusted" reading (calculated by subtracting the reading from a negative well; readings below .02 were considered too low to quantify) were removed from analysis, leaving only 10 quantifiable ratios. For each sample, an average of the two wells was taken for computing the total readout; all samples were normalized to a reading and parasitemia for 1 mL of solution. Geomeans of urine and plasma protein concentrations, geomeans of ratios of concentrations (plasma to urine), as well as the ratio of the geomeans of plasma to urine concentration were computed.

Nanoparticles

Nanoparticles and all Western Blots and analysis pertaining to their use were generated at George Mason University in Manassas, Virginia under the guidance of Drs. Lance Liotta and Alessandra Luchini.

Nanoparticles themselves were manufactured by other members of the lab. After testing with nanoparticle functionalized using different dyes, "Remazol Brilliant Blue" particles were selected (see Results) and manufactured as previously described [65].

For preliminary determination of optimal nanoparticle and nanoparticle quantity, and as a control when running samples, urine spiked with recombinant HRPII protein was used. Fresh urine was captured and spun for 15 minutes at speed to remove excess proteins, then calibrated to pH 8.0. Specific quantities of recombinant *Pf*HRPII protein was added prior to use in nanoparticle incubation process.

To capture antigen with the nanoparticles, 500 microliters of spiked urine HRPII or patient urine samples were incubated with 150 microliters of nanoparticles at room temperature on a rotator for one hour, then spun at 16,100 rcf for 15 minutes. The supernatant from this spinning step was placed in a separate Eppendorf tube and saved

for analysis via Western Blot (to determine how much of the protein in question was . The nanoparticle pellet was then washed in 1 mL of water and fully resuspended, then spun again for 15 minutes at 16,100 rcf. The wash solution was removed and discarded, and the nanoparticle pellet was resuspended in 35 microliters of 2X Sample Buffer (Bio-Rad) with a 1:20 dilution of TCEP and placed on a 95 degree heat block for 10 minutes. These were then centrifuged at 16,100 rcffor 25 minutes; the supernatant from this final spin was transferred to Eppendorf tubes labeled “eluate” and analyzed using Western Blots.

Western Blot protocols undertaken at George Mason University differed slightly from those at Johns Hopkins School of Public Health. 4X sample buffer (Bio-Rad) with TCEP was used to denature proteins for 10 minutes at 100 degrees. For samples and supernatants, 15 microliters were combined with 5 microliters of loading buffer and boiled for 10 minutes at 100 degrees. For eluates from nanoparticle incubation, boiling was performed as described prior to the final centrifugation step, and yielded approximately 30 microliters of final solution. These samples were run on Novex 4-20% Tris-Glycine gels (Invitrogen) for 45 minutes at 200 mV using Tris-Glycine SDS running buffer in a Novex X-Cell IITM Mini-Cell (Invitrogen). Transferring was performed in the Novex Mini-Cell (Invitrogen) in transfer buffer (made with 3.03 grams Tris-HCl, 14.4 g glycine, 200 mL methanol and 1 mL 10% SDS brought to 1 liter of solution with deionized water**)** for 105 minutes at 25 mV onto PVDF membranes (Millipore) that were soaked for one minute in methanol prior to use. Membranes were then blocked in 10 mL of I-Block (Applied Biosciences) solution for one hour on a shaker; this was then discarded, and 10 mL 0.2% I-Block (Applied Biosciences) with 0.1% Tween 20 (Fisher) plus 10 uL of 3A4 antibody was added for 90 minutes on a shaker. Subsequently, blocks were subjected to 5, five-minute washes in 0.01% PBS-T before being placed in 10 mL

of I-Block with 3 mL of anti-mouse secondary antibody for an hour at room temperature on a shaker. After this final incubation period, blots were washed 5 more times in 0.01% PBS-T for 5 minutes each before imaging. Proteins were detected using chemiluminescence (SuperSignal West Dura, ThermoFischer Scientific) on a Kodak MM4000 Imager.

Analysis

All analysis was done in GraphPad Prism and Stata. Logistical regression analysis was used to determine the change in odds of positive results on various urine-based diagnostic tests (i.e. dot blot, western blot, ImmunoPCR, and ELISA) using criteria such as blood parasitemia, protein level in the urine, and presence or absence of fever. All analysis was undertaken using the assumption that thick-film microscopy continues to be the “gold standard” for malaria detection.

Results

Patient characteristics

A total of 202 urine samples were available for analysis. Of the patients from which this urine was procured, 16 had parasites/ul >= 100,000, 43 = pars/ul 5,000-99,999, 43 had mild malaria with less than 500-5,000/ul and 28 had less than 500/ul. 72 had no malaria on the blood film (Figure 2). A total of 29 urine samples contained traces of blood. Parasitemia was categorized for analysis into the four aforementioned categories (no parasitemia, 500 to 4,999 parasites/mL, 5000-99,999 parasites/mL, and >=100,000 parasites/mL) for analysis. In total 27 persons were febrile with 7 in the nonparasitemic group.

Table 1 shows counts of patient samples divided by microscopic parasitemia and other diagnostic criteria, with a subset divided by febrile status. Microscopy was considered the “gold standard” for malaria in all analyses, despite the fact that ImmunoPCR may be more sensitive.

Western Blot analysis

Samples positive by microscope as the “gold standard” for which we had 10 microL of urine were analyzed via Western Blot, along with negative controls. In total, 74 microscopy-positive and 12 microscopy-negative samples were run, for a total of 86 samples (Table 1**)**. Figure 3 shows a representative sample Western Blot. Of the 74 microscopy-positive, 19 were positive by Western Blot, yielding approximately a 25.7% sensitivity. 12 microscopy-negative urine samples were also run, all of which gave negative results, for a specificity of 100%. A total of 3 Western-positive samples contained traces of blood, while 10 Western-negative samples contained blood. Comparing the Western Blot of urine to the “gold standard” of microscopic parasitemia showed a 36.0% agreement.

Analyzing by febrile status, 6 of 17 (35.3%) microscopy-positive with fever were Western-positive, while only 13 of 67 (22.8%) of afebrile, microscopy-positive patients were Western-positive (Table 1).

Regression analysis yielded a statistically significant relationship between odds of positive Western Blot results and proteinuria below 600, parasitemia (as a categorical variable), but a nonsignificant relationship with fever and proteinuria above 600, when taken individually. Each additional level of parasitemia was associated with 2.8 times the odds of a positive Western result (95% CI: 1.37 to 5.56, $p<0.01$) when taken alone. When controlling for proteinuria and fever, this relationship was attenuated and still statistically significant, with an odds ratio of 2.16 (95% CI 1.05-4.48, $p<0.05$) for each increase in parasitemia level. Proteinuria was only statistically significantly related to odds of positive Western results below 600 mcg/mL of protein, with a 0.1% increase in odds for each unit increase in protein content of the urine (95% CI 1.001-1.008, $p=0.01$). Fever was not statistically significantly correlated with odds of Western Blot positivity, but attenuated the relationship of categorical parasitemia level with Western Blot positivity.

Dot blot analysis

All 202 urine samples were analyzed by dot blot again with 10 microL of urine; the subset for which ELISA was performed also had plasma analyzed by dot blot. Figure 4 shows a representative dot blot for urine, and figure 5 shows the dot blot for plasma. Dot blot analysis was more sensitive than Western Blot analysis, but less specific. Overall, 51 of the 130 patients with microscopic-level parasitemia were positive by dot blot, implying a 39.2% sensitivity. Of the 21 febrile patients with malaria, 10 (or 48%) were Dot blot positive; for the 110 afebrile patients, 42 (38%) were Dot blot positive. Of those without microscopic malaria, 59 out of 72 did not have visible *Pf*HRPII by Dot blot; this

implies a specificity of 80%, with 13 false positives. Ten samples that were dot blot positive contained traces of blood, while 19 samples that were dot blot negative contained traces of blood.

Univariate logistic regression analysis yielded a statistically significant relationship between odds of positive dot blot and categorical parasitemia (OR = 1.33, 95% CI = 1.07-1.67, $p<0.05$), and proteinuria below 600 (OR = 1.003, 95% CI 1.001-1.005, $p=0.005$), but no significant relationship existed for proteinuria above 600 or for fever. In multivariate analysis, categorical parasitemia no longer statistically significantly predicted dot blot positivity (OR = 1.18, CI = 0.93-1.51, $p=0.17$) when controlling for proteinuria and parasitemia.

Buffered urine-specific (BUS) RDT

Of the 130 microscopy-positive individuals whose urine was tested with the buffered urine-specific RDT, 42 had a positive result, yielding a 32.3% sensitivity. Of the 72 microscopy-negative samples analyzed, 1 yielded a positive BUS RDT result, yielding a 99% specificity. Overall, the BUS RDT had a 55.9% agreement with microscopy, higher than both Western blot and dot blot, making it the highest of the three urine-detection methods used. There was a 76.7% agreement with the Western Blot, with the BUS RDT as the more sensitive test (McNemar's test, OR = 3, 95% CI=1.04-10.56, $p<0.05$). Comparing to the dot blot, the BUS RDT had a 70.8% agreement, but the BUS RDT was less sensitive (McNemar's test, OR = 0.475, 95% CI 0.260-0.840, $p<0.01$).

Among those with microscopic malaria and fever, the BUS RDT yielded a positive result in 60% of cases, whereas afebrile, microscopy-positive cases had positive BUS results in only 24% of cases.

Univariate logistic regression analysis demonstrated a statistically significant relationship between odds of positive BUS RDT and categorical parasitemia (OR=2.86, 95%CI=

2.04-4.02, p<0.001), proteinuria below 600 (OR = 1.006 per unit increase in proteinuria, 95% CI = 1.003-1.008, p=<0.001), and fever (OR=3.71, 95%CI = 1.58-8.71, p<0.01), but nonsignificant relationships with proteinuria above 600 (OR=0.998, 95%CI = 0.994-1.002, p=0.40).

Multivariate logistic regression analysis attenuated all these relationships, but they remained significant or bordering on significant. Each increase in microscopic parasite level was associated with a 2.56 times higher odds of BUS RDT positivity (95% CI 1.80-3.72, p<0.001), and each unit (micrograms/mL) increase in proteinuria below 600 micrograms/mL was associated with a 0.4% increase in odds of positive BUS RDT (95% CI 1.001-1.006, p<0.001). Having a fever was associated with 2.94 times the odds of positive BUS RDT, but this relationship was borderline nonsignificant when controlling for other variables (95% CI = 1.00-8.7, p=0.052).

Aim 2:

ELISA quantification of *Pf*HRPII in urine and plasma samples

A subset of microscopy-positive samples for which ImmunoPCR analysis had been performed and for which plasma and samples were available were selected for analysis. Preliminary ELISA analysis on some of these urine samples yielded undetectable amounts of urine *Pf*HRPII; these samples were removed from consideration, as they would skew the ratio to make it appear higher. In the final ELISA, twenty matched pairs of plasma and urine were analyzed; 10 of these yielded detectable, quantifiable *Pf*HRPII concentrations in urine (the limiting factor, as it had lower concentratins) and thus yielded calculable ratios. Of these final 10, 2 were febrile and 8 were afebrile (Table 1). Figure 6 shows the concentrations of *Pf*HRPII in the matched urine and plasma samples as determined by ELISA, along with descriptive statistics. The geometric mean of the concentration of *Pf*HRPII in plasma was 1732.0 (95% CI: 743.1-4036.9) while that of

*Pf*HRPII in urine was 25.1 ng/mL (95% CI: 4.8 to 130.8). This data is statistically significantly different, with a median difference of -1522 ng/mL between plasma concentration of *Pf*HRPII and urine concentration of *Pf*HRPII (Wilcoxon paired t-test, p=0.002). The geometric mean of the ratios was 69.0 (95% CI: 16.8-282.6), which was the same as the ratio of geometric means.

ImmunoPCR quantification of *Pf*HRPII in urine and plasma samples

70 matched plasma and urine samples had previously been analyzed by ImmunoPCR for quantity of *Pf*HRPII. Of these, 36 had microscopy-confirmed malaria (with 4 febrile patients), while 34 did not (with 2 febrile patients; see Table 1). ImmunoPCR results were dichotomized into "positive" and "negative" with a cutoff of 1 ng/mL as the minimum quantity of *Pf*HRPII detection necessary for a "positive" result (see Table 1). Figure 7 shows concentrations of *Pf*HRPII in the matched urine and plasma samples of microscopy-positive patients; including microscopy-negative patients would unnecessarily skew the data. There was a statistically significant difference in *Pf*HRPII concentration between the two body fluids, with an average difference of -21360 ng/mL between plasma and urine (Paired t-test, 95% CI: -35660 to -7055, $p<0.05$). The geometric mean concentration in plasma was 1439.06 ng/mL, while that in the urine was 10.5 ng/mL. The geometric mean of the ratio was 137.6 (95% CI: 54.2, 349.0; Table 2), while the ratio of the geometric means was 137.1.

When ImmunoPCR was dichotomized into "positive" or "negative" for urine detection (with a threshold of 1 ng/mL), 24 were positive among 36 with microscopic malaria, implying a sensitivity of 66.7% (Table 1). Univariate logistic analysis yielded a statistically significant relationship between odds of ImmunoPCR detection and categorical parasitemia (OR=2.20, 95% CI= 1.36-3.5, $p<0.01$), proteinuria below 600 (OR=1.009, 95%CI = 1.004-1.01, $p<0.01$), proteinuria above 600 (OR=0.99, 95%CI =

0.989-0.999, p<0.05), but not fever (OR=1.13, 95%CI=0.21-6.04, p>0.5). Multivariate logistic analysis maintained a statistically significant relationship between categorical parasitemia and odds of ImmunoPCR detection (OR=1.79, 95%CI=1.06-3.01, p<0.05) and proteinuria below 600 (with a .7% increase in odds per unit of proteinuria, 95% CI =0.2-1.3, p<0.01) but no relationship with proteinuria above 600 or fever.

Analysis was then subdivided into those with malaria by microscopy (deemed the gold standard for this analysis) and those without. Table 2 shows *Pf*HRPII ratios for those with and without malaria. Among those with microscopic malaria, the ratio of *Pf*HRPII in plasma versus urine had a geometric mean of 134.3 (95% CI: 54.3-332.4). For the four febrile patients, the geometric mean ratio was 80.6 (95% CI: 1.6-4007.2), while for the 32 afebrile patients the geometric mean ratio was 147.1 (95%CI: 53.3 to 405.8). The small sample size and large variation prevented this difference from achieving statistical significance.

Comparison of ratios determined by ELISA and ImmunoPCR over all microscopy-positive analyzed samples showed no statistically significant difference (Figure 8). Subsequent analysis was done on the subset of samples for which both ELISA and ImmunoPCR data was collected (Figure 9); this consisted of 9 samples, all of which were microscopy-positive for malaria. The mean geometric ratio of *Pf*HRPII concentration as determined by ImmunoPCR was 26.7 (95% CI: 4.47-159.8), while that determined by ELISA was 55.9 (95% CI: 12.3 to 254.0); there was no statistically significant difference between the two.

Figure 10 shows a Western Blot and Coomassie Brilliant Blue stain of matched urine-plasma pairs. Equal volumes of urine and plasma were used within the pair set, and the rough equivalence of intensity in bands shows a rough 1:100 dilution factor of protein (based on the 1:100 dilution of the plasma samples).

Aim 3

Nanoparticle selection and optimization

Figure 11 shows initial steps in choosing the appropriate nanoparticle for *Pf*HRPII concentration using 500 microliters of samples (in this case, urine from healthy volunteers spiked with recombinant *Pf*HRPII). Higher protein content in the eluate (i.e. protein captured in the nanoparticle) as well as low protein content in the supernatant (i.e. protein left behind in solution and not trapped by the nanoparticle) led to the selection of nanoparticle D, or Remazol Brililant Blue nanoparticles.

Figure 12 shows a dilution curve of five-fold dilutions using malaria-negative urine spiked with recombinant *Pf*HRPII. ImageJ analysis yielded a six-fold change in band intensity (i.e. protein content) between the initial sample and the eluate. The limit of detection was approximately 0.57 ng/mL, with a theoretical total of 0.285 nanograms in 500 microliters (Figure 12).

In total, nine samples were tested with the nanoparticles; six of these had previously yielded positive results on a Western Blot, and three were microscopic parasitemia-negative controls. Figure 13 shows an example Western Blot run with samples. *Pf*HRPII was only detected in the eluate of one sample, Sample 7 (see Figure 13). All other samples did not yield detection either with or without nanoparticle concentration.

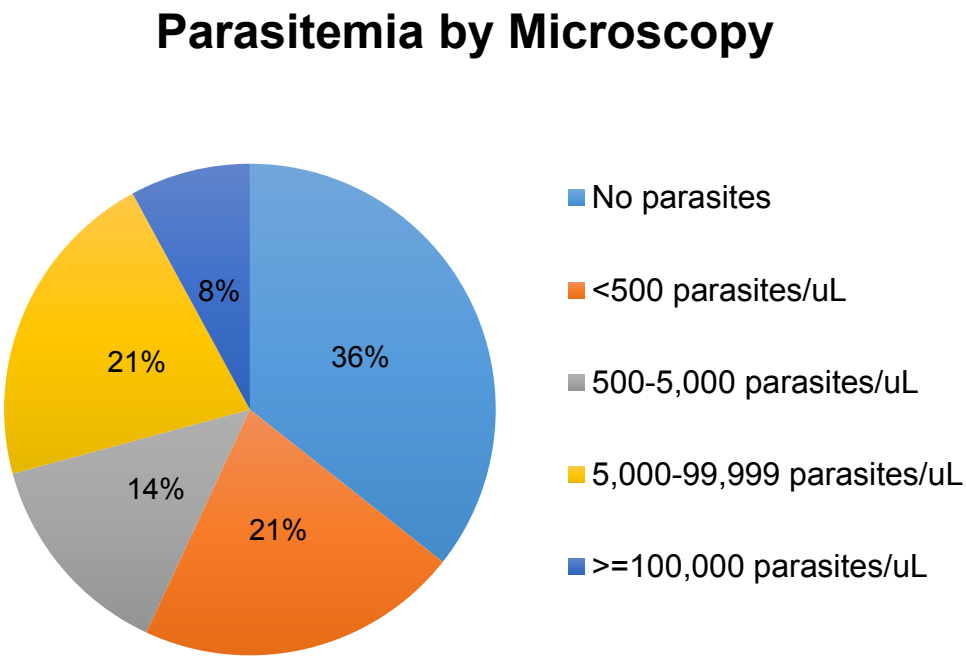
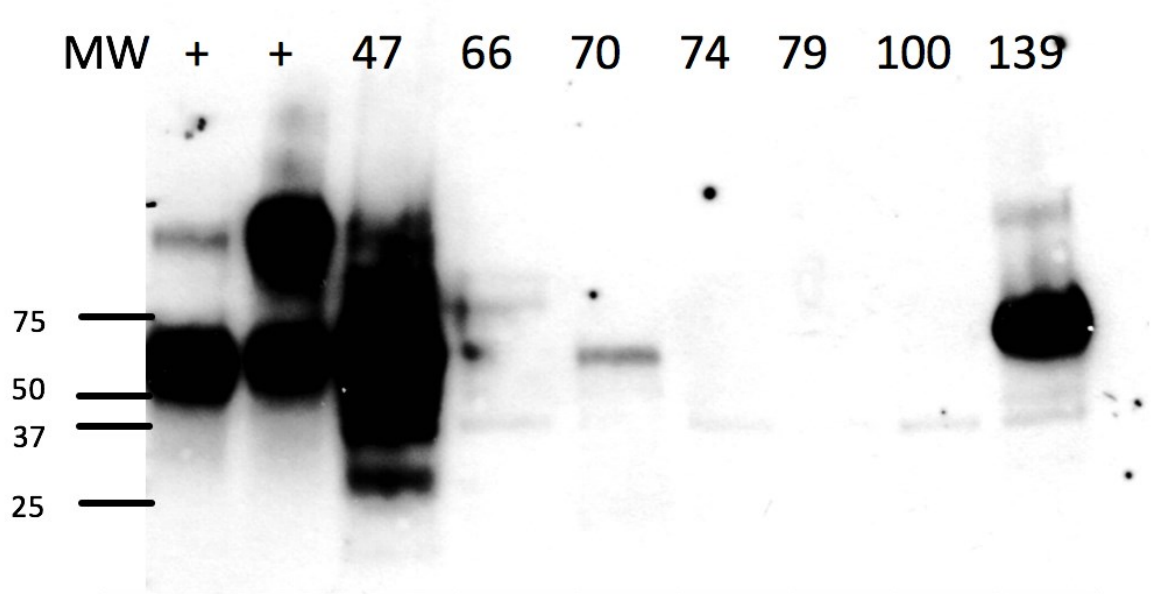


Figure 2. **Distribution of microscopic parasitemias of Ghanaian patient samples.** All parasitemias determined by thick smear blood film.

	Microscopy +	Microscopy -	Microscopy +		Microscopy -	
			Febrile	Afebrile	Febrile	Afebrile
Total (n=202)	131	71	20	110	7	65
Western (n=86)						
Western +	19	0	6	13	0	0
Western -	55	12	11	44	0	12
Dot blot (n=202)						
Dot blot +	51**	13**	9	42	3	10
Dot blot -	79**	59**	11	68	4	55
IPCR (n=70)						
IPCR +	24**	9**	3	21	0	9
IPCR -	12**	25**	1	11	2	23
ELISA (n=10)						
ELISA +	10	0	2	8	0	0
ELISA -	0	0	0	0	0	0
BUS (n=202)						
BUS RDT +	42**	1**	12**	30**	0	1
BUS RDT -	88**	71**	8**	80**	7	64

Table 1. Sample counts and positive results for various diagnostic techniques detecting PfHRPII in urine. ImmunoPCR (IPCR) positive was deemed as having any reading over 1 ng/mL. BUS denotes buffered urine-specific test, manufactured by Binax. "Gold standard" was considered to be blood smear microscopy. Fisher's exact test (two-tailed) was used to compute statistical differences in group sizes comparing proportion of positive results on all listed urine-based detection methods comparing microscopy + to microscopy -, or febrile versus afebrile within microscopy categories. * denotes $p<0.05$, ** denotes $p<0.01$, all unmarked are not statistically significant.



BUS RDT	1	0	1	1	0	1	1
Fever	1	0	0	1	0	1	0

Figure 3. Representative Western Blot for detection of PfHRPII in 10 microliters of Ghanaian urine samples. (+) lanes indicate positive controls using recombinant *Pf*HRPII; all other lanes correspond to microscopy-positive patient samples.

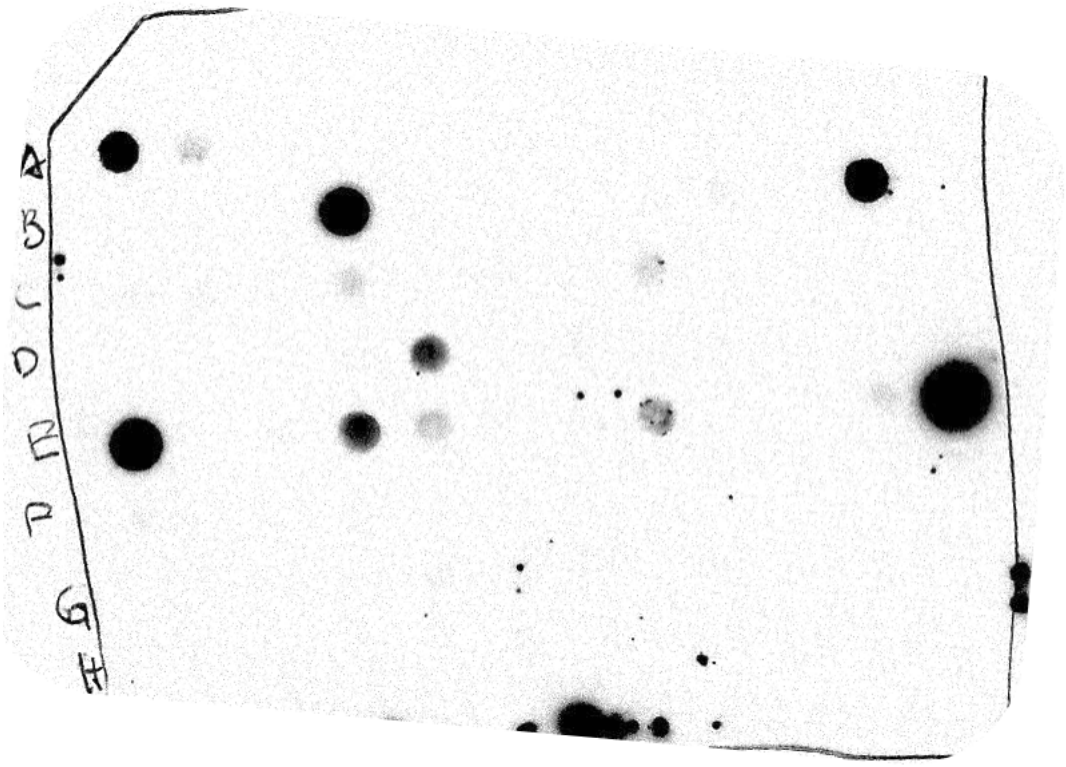


Figure 4. Representative dot blot for detection of PfHRPII in 10 microliters of Ghanaian urine samples. First three spots represent a standard curve, while the rest are all patient samples.

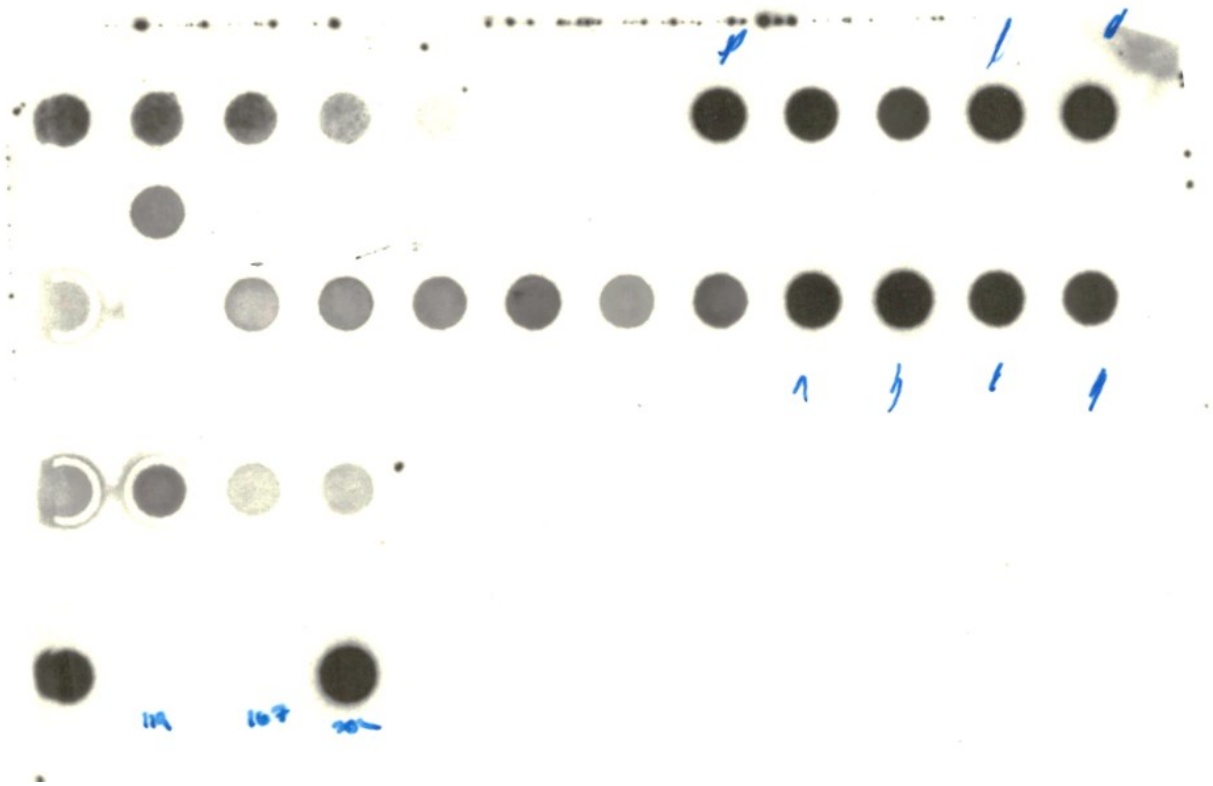
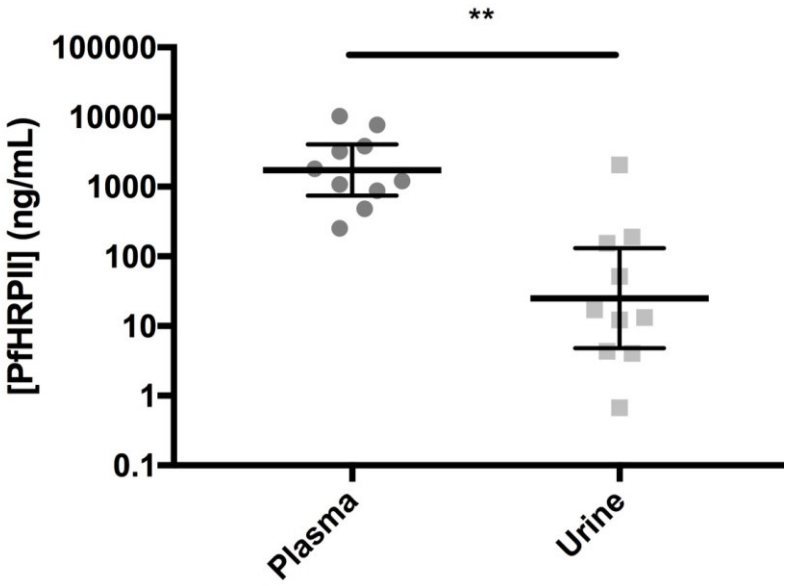
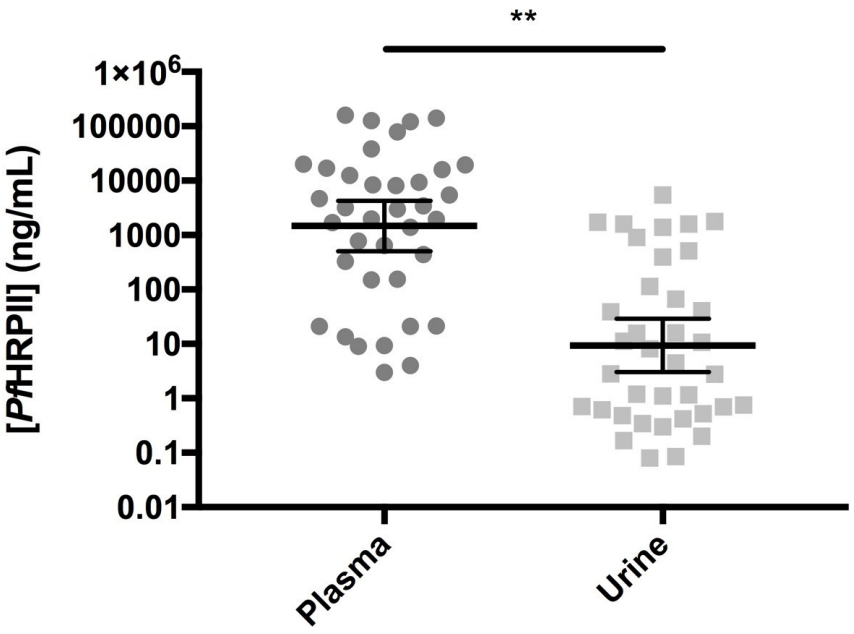


Figure 5. Representative dot blot for detection of PfHRPII in 10 microliters each of Ghanaian plasma and urine samples. First six spots represent a standard curve, while last four are urine samples for comparison with other blots. All plasma samples are diluted 1:100.



	Plasma (ng/mL)	Urine (ng/mL)	Ratio
Max:	10240.00	2053.40	714.93
Min:	253.00	0.67	4.61
Ave:	3073.35	249.98	224.66
Median:	1507.00	15.14	155.05
Geomean:	1732.00	25.11	68.97
STD:	3374.71	637.22	249.63

Figure 6. Comparison of paired plasma and urine samples from microscopy-positive Ghanaian patients (n=10) detecting PfHRPII using ELISA. Analysis shows a statistically significant difference in *Pf*HRPII concentrations between the two fluids and a geometric mean ratio of plasma:urine of 68.97. Graph also displays 95% confidence intervals of geometric mean. Patients were all positive by microscopy (the gold standard for this analysis).



	Plasma (ng/mL)	Urine (ng/mL)	Ratio
Max:	158711	5490.40	54690.41
Min:	2.98	0.08	1.22
Ave:	22306.43	437.19	.2942.32
Median:	2592.39	6.30	62.58
Geomean:	1439.06	10.5	137.57
STD:	43960.18	1038.15	9339.58

Figure 7. Comparison of paired plasma and urine samples from microscopy-positive Ghanaian patients (n=36) detecting PfHRPII using ImmunoPCR techniques. Analysis shows a statistically significant difference (Paired t test, $p<0.01$) in *Pf*HRPII concentrations between the two fluids and a geometric mean ratio of plasma:urine of 58.06. Patients were all positive for malaria by microscopy (the gold standard).

	All samples	Microscopy +	Microscopy −	Microscopy +		Microscopy -	
				Febrile	Afebrile	Febrile	Afebrile
Count	n=202	n=130	n=72	n=20	n=110	n=7	n=65
Geomean urine protein content, mcg/mL (95% CI)	101.9 (86.3-120.3)	122.0 (98.9-150.6)	73.6 (56.9-95.3)	165.8 (97.3-282.7)	115.4 (91.6-145.4)	139.8 (79.0-247.4)	68.7 (52.1-90.7)
Geomean parasitemia, parasites/uL (95% CI)	-	2358 (1436-3872)	-	8028 (2502-25753)	1887 (1096-3249)	-	-
ImmunoPCR							
Count	n=70	n=36	n=34	n=4	n=32	n=2	n=32
Geomean urine [*Pf*HRPII], ng/mL (95%CI)	-	10.5 (3.4-32.6)	0.60 (0.39-0.94)	41.7 (0.1-18363)	8.8 (2.7-29.1)	0.2 (0.02-2.14)	0.7 (0.4-1.0)
Range urine [*Pf*HRPII], ng/mL	0.04-5490.4	0.08-5490	0.04-19.4	0.42-1728	0.08-5490	0.17-0.24	0.04-19.4
Geomean plasma:urine [PfHRPII] ratio (95% CI)	-	137.6 (54.2-349.0)	22.5 (12.7-39.8)	80.6 (1.6-4007.2)	147.1 (53.3-405.8)	886.0	17.9 (11.8-27.1)
Range plasma:urine [*Pf*HRPII] ratio	1.2-54690	1.2-54690	2.9-18152.3	5.4-2073	1.2-54690	43.2-18152.3	2.9-584.1

Table 2. Comparison of total urine protein content, urine *Pf*HRPII content, plasma;urine *Pf*HRPII ratio, and parasitemia by malaria status and febrile status.

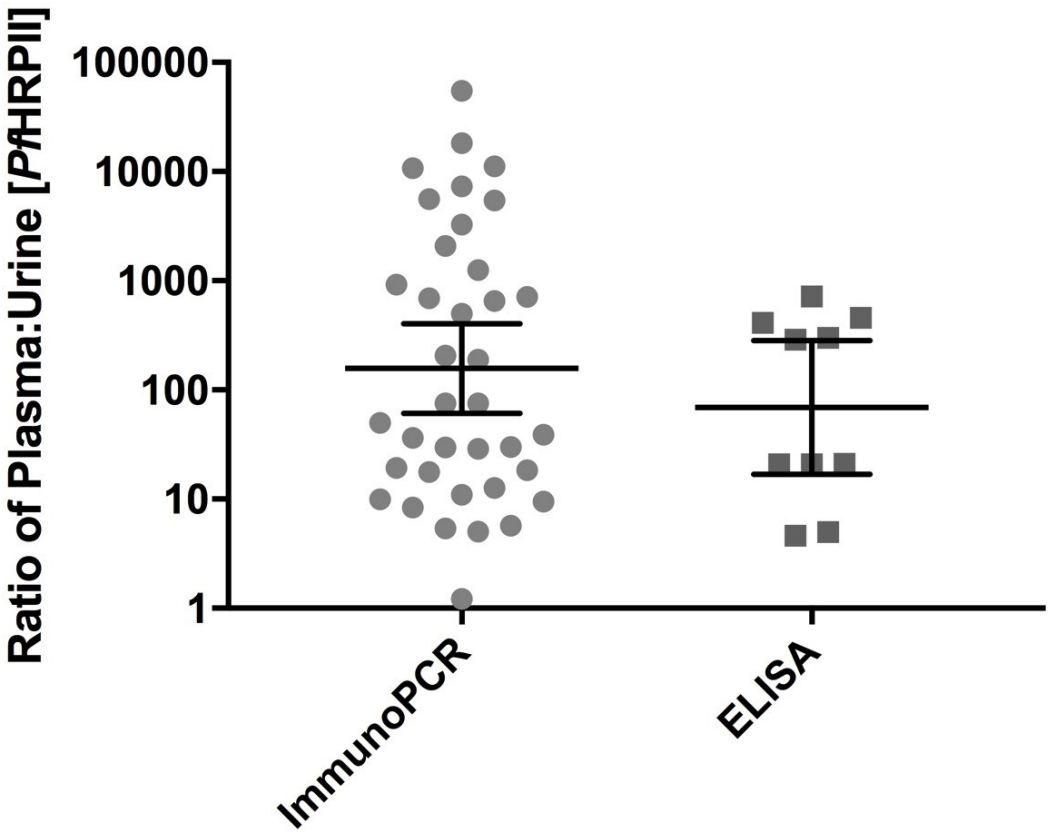


Figure 8. Comparison of ratios of plasma-to-urine concentration of PfHRPII as measured by ImmunoPCR (n=36) or ELISA (n=10). Whiskers represent 95% confidence intervals, demonstrating statistical equivalence.

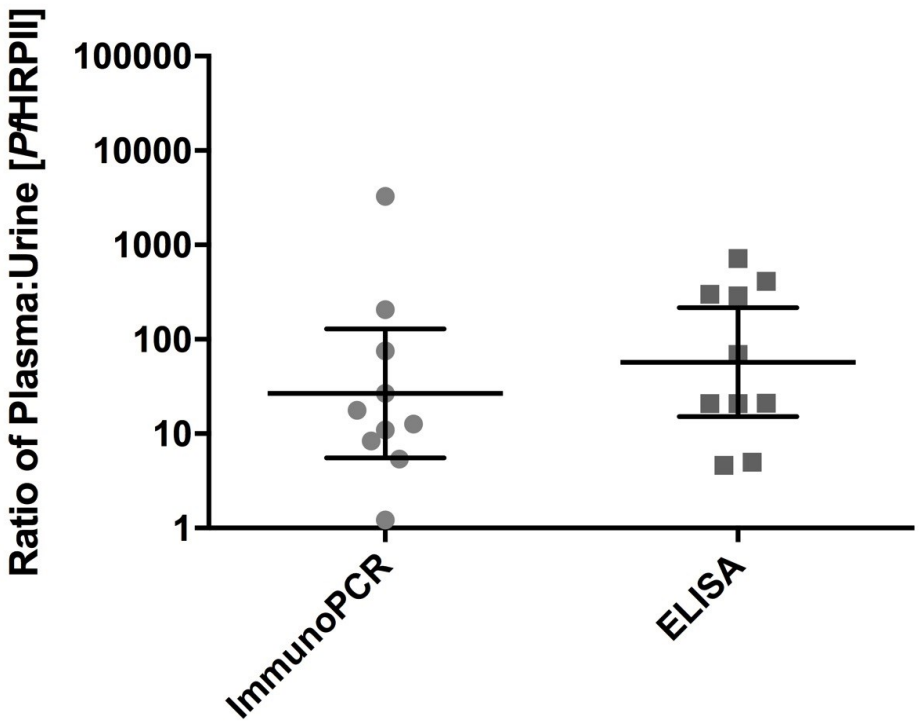
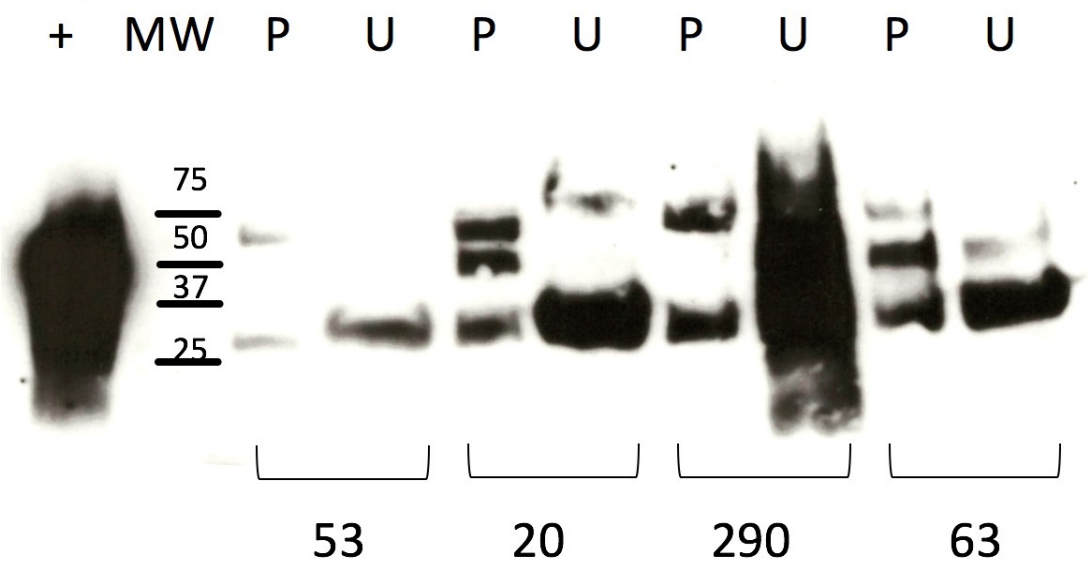


Figure 9. Comparison of plasma-to-urine PfHRPII ratios determined by ImmunoPCR and ELISA for samples analyzed by both techniques (n=9). Whiskers denote 95% confidence intervals and demonstrate statistical equivalence.

a.



b.

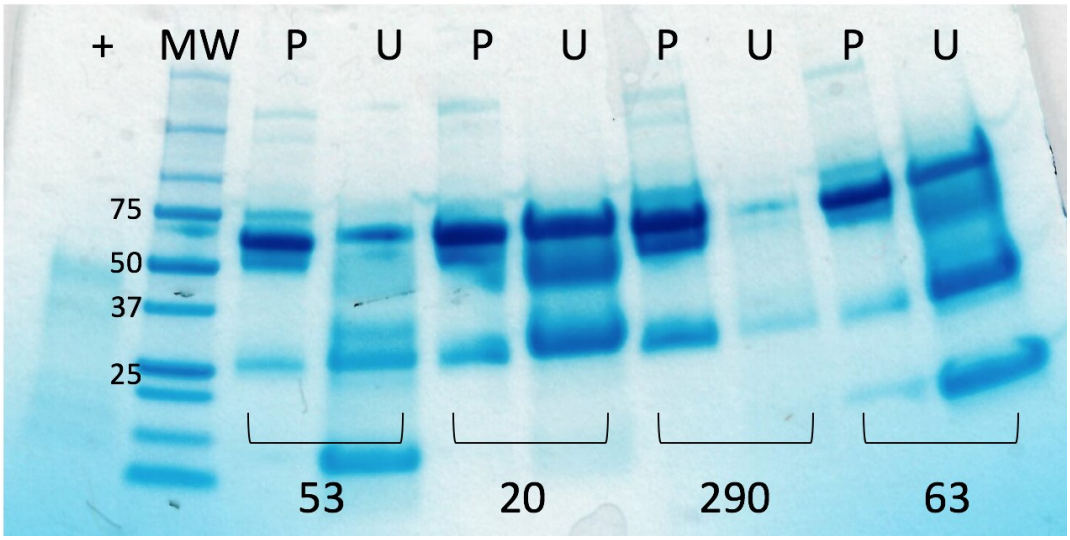


Figure 10: Visual comparison of 1:100 dilution of plasma (P) to full samples of urine (U) in matched samples from Ghanaian patients using Western Blot (a) and Coomassie Brilliant Blue (b). Band intensities are roughly equivalent between pairs, implying a roughly 100-fold dilution in PfHRPII concentration in urine versus plasma. Western Blot(a) and CBB stain(b) of matched pairs of plasma (P) and urine (U) from *P. falciparum*-infected patients in Ghana. All plasma samples were diluted 1:100 prior to use. Lanes for samples 53, 20, and 63 contain 24 uL of sample, while lanes for sample 290 contain 21 uL.

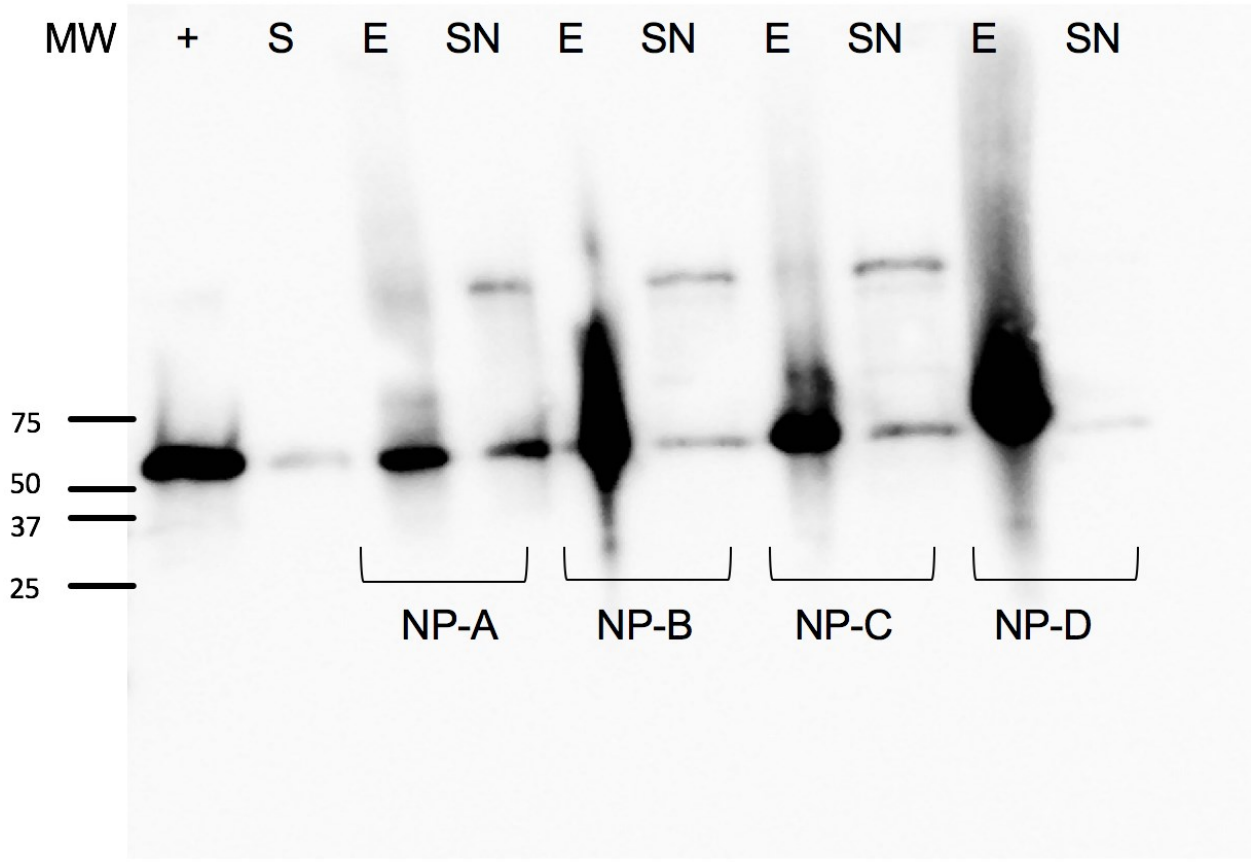


Figure 11. Nanoparticle selection. Nanoparticle D is most efficient at concentrating *Pf*HRPII from malaria-negative urine samples spiked with recombinant *Pf*HRPII. *Pf*HRPII was spiked in 500 µL of human urine from healthy volunteers (S) and incubated with Nanotrap particles functionalized with different dyes. After particle processing, *Pf*HRPII in an optimal Nanoparticle should be depleted from the supernatant (SN) and easily detected in the eluate (E) to show nanoparticle capture. Lane 1 is a molecular weight ladder; Lane 2 is a positive control; Lane 3 shows 15 microliters of the original diluted sample, Lanes 4-5 are eluate and supernatant for Nanoparticle A, Lanes 6-7 are eluate and supernatant for Nanoparticle B, Lanes 8-9 are eluate and supernatant for nanoparticle C, and Lanes 10-11 are eluate and supernatant for Nanoparticle D. Nanoparticle D (Remazol Brilliant Blue) shows highest concentration of captured *Pf*HRPII in the eluate (E) and least *Pf*HRPII in the supernatant (SN).

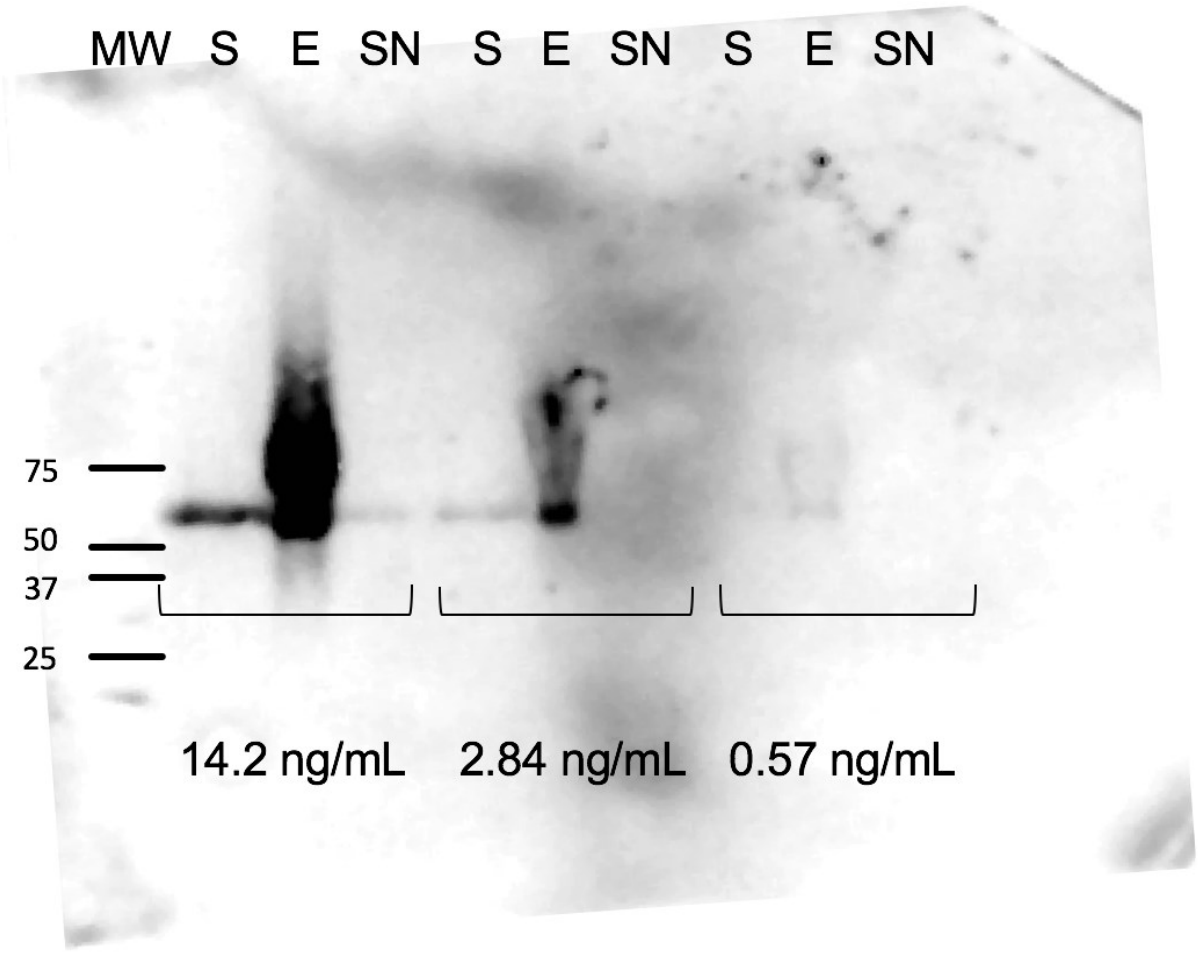


Figure 12. Dilution curve using nanoparticle concentration to determine limit of detection using 500 microliter samples. The limit of detection for nanoparticle-concentration of 500 microliters of urine spiked with recombinant *Pf*HRPII is 0.57 ng/mL (285 ng in sample). Different quantities of recombinant *Pf*HRPII were spiked into the urine of healthy volunteers and diluted sequentially 1:5. Lanes marked "S" contain 15 microliters of original diluted samples corresponding to the concentration listed below the blot. Lanes marked E contain eluate from 500 microliters of sample at the concentration listed below incubated with Remazol Brilliant Blue nanoparticles. Lanes marked "SN" contain 15 microliters of left over supernatant from nanoparticle protocol corresponding to sample with concentration listed below to show protein not captured in the nanoparticle.

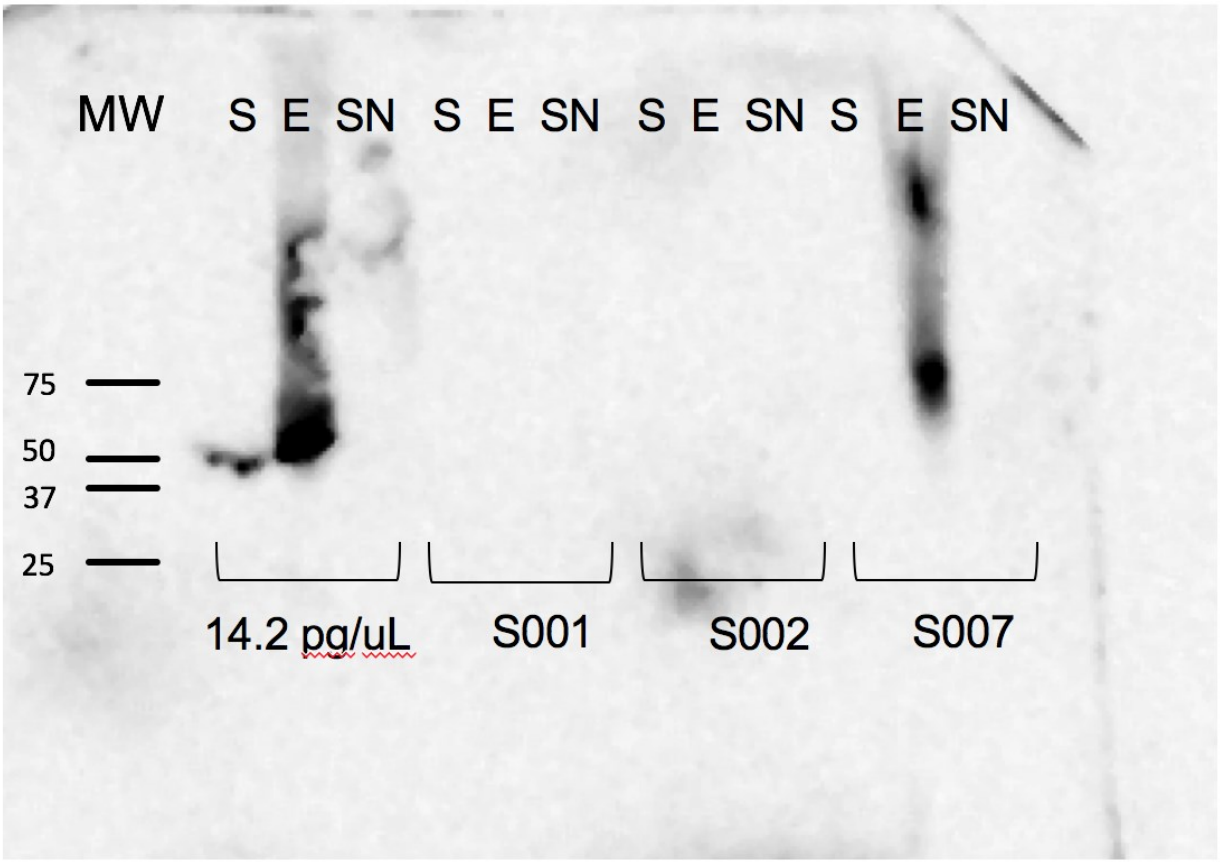


Figure 13. Testing of nanoparticles for concentration of PfHRPII in clinical samples. Incubation of Ghanaian urine samples with Nanotrap particles was suboptimal for all but one sample**.** Western Blot performed for detection of *Pf*HRPII, with a recombinant control in lanes 2-4 and patient samples in lanes 5-12. (S) denotes original sample, (E) denotes eluate (i.e. *Pf*HRPII protein captured inside nanoparticle), and (SN) denotes supernatant (i.e. PfHRPII protein not captured inside nanoparticle). Of all 10 samples tested, only S007 demonstrated any presence of protein in eluate or sample.

Discussion

This work demonstrates some of the challenges present in creation of urinary malaria diagnostics. Both the dot blot and Western Blot themselves had low sensitivity with 10 microL analyzed, with the dot blot suffering additionally from hampered specificity. Although ImmunoPCR also had imperfect specificity, this could in theory be an artifact of increased sensitivity. However, it also had a 66% sensitivity as compared to microscopy and thus would miss some cases if used as the "gold standard". The buffered urine-specific test used about 50 to 70 microL and was more sensitive than the Western Blot but less sensitive than the dot blot, although it was 99% specific versus 80% specific. The BUS-RDT had higher sensitivity among those with fever and malaria, correctly identifying 60% of malaria cases in febrile malaria patients versus 37.5% in afebrile patients.

Among all microscopy-positive samples tested, the quantity of *Pf*HRPII has a geometric mean of 25.11 ng/mL and a median of 15.1 ng/mL by ELISA (Figure 6); with a geometric mean of 10.5 ng/mL and a median of 6.30 ng/mL by ImmunoPCR (Figure 7);, The lower immunoPCR number is from inclusion of many more low *Pf*HRPII samples in immunoPCR near the level of detection by immunoPCR, not detected by ELISA. Considering all 70 samples (microscopy positive and negative), ImmunoPCR yields a geometric mean plasma to urine ratio of 57.1; however, this is likely to be skewed by negative points. When including malaria-negative samples, ImmunoPCR yields a much lower mean and median; this discrepancy is due to the inclusion of presumably negative samples in the overall analysis, so analysis was primarily done on malaria-positive patients. ELISA shows an approximate geometric mean plasma to urine ratio of 69. However, none of these appear to be statistically significantly different. Figure 10 demonstrates visually that the quantity of *Pf*HRPII in 100-fold dilution of plasma is

approximately equal to that of its undiluted matched urine sample. Taken all together, we can round to an approximate 100-fold higher concentration of *Pf*HRPII in plasma as compared to urine by these ratios.

In addition, as mentioned, the geometric mean quantity of *Pf*HRPII in urine was 25 ng/mL using an ELISA technique and 10.5 ng/mL using ImmunoPCR (Figures 6 and 7); to reach the approximate functional limit of detection of 200 ng/mL, this would require 10-100 times more sample volume or a test with 10-100 times greater sensitivity. However, there still exists an enormous amount of variability in the quantities of *Pf*HRPII detected as seen in the very wide ranges (Figures 6 and 7, Table 2). This implies that basing our needs on the geomean may still yield inadequate overall sensitivity. With an 100-fold increase, 15/36 (or 40.5%) of microscopy-positive samples are still below the 200 ng/mL threshold using quantities detected by ImmunoPCR, yielding a sensitivity of 59.5%. With a 200-fold increase, 12/36 (33.3%) will be difficult to detect, and even at a 500-fold increase 5/37 (13.8%) will remain below the assumed 200 ng/mL limit of detection, with roughly 87.5% sensitivity. Among those analyzed by ImmunoPCR and microscopy-positive for malaria, the minimum value of *Pf*HRPII concentration is 0.08 ng/mL. By this metric, in order to detect all samples at 200 ng/mL, we would need a 2500-fold increase in sample volume. Given that most blood-based RDTs use 10 microliters of blood, this would entail a 25 mL sample.

Based on our analysis of the capabilities of Nanotrap technology using 500 microliters of sample, the nanoparticles are still inadequate to overcome this barrier of dilution, as only one patient sample was successfully concentrated (Figure 13). Greater sample quantities (as used in the Lyme test, for instance) may help overcome this apparent failure; as discussed above, a much larger increase in sample size (at least 2500-fold) would be necessary to mitigate the decreased concentration of *Pf*HRPII in urine. Even so, proteins that were successfully captured and concentrated from 500

microliters in Nanotrap technology showed only a 6-fold increase in band intensity for *Pf*HRPII as compared to identical 15 microliter samples run without Nanotrap (Figure 12), which is much less than the 31-fold increase in sample quantity, implying an imperfect capture of antigen. This could potentially be rectified through changes to the specific dye used as bait within the nanoparticle, as higher affinity dyes are continuing to be discovered.

This around-100-fold ratio is also much lower than that typically observed of normal proteins in normal patients. For normal patients without fever, blood protein levels are typically 400 times urine protein levels. This is likely due to an overall increase in protein shedding. Infection with malaria[67] and fever[68] have both independently been found to be associated with proteinuria in the past. One study found that 78% of malaria cases observed experienced transient proteinuria, even in the absence of overt renal failure[69]. Among malaria-infected patients, fever appears to be associated with greater proteinuria[67], and level of proteinuria may be correlated (albeit not statistically significantly) with parasitemia[70]. We also found that the ratio of *Pf*HRPII concentration in plasma to that in urine was only 80 for febrile patients, versus 147 in afebrile patients (Table 2), again implying a role of fever in aiding detection (although due to small sample size, the difference was not statistically significant).

This would imply that in our study, febrile patients would be more easily diagnosed. 28% of febrile patients with microscopic malaria were positive by Western, versus 11% of afebrile patients (Table 1). 60% of malaria-infected, febrile patients were positive by buffered urine-specific RDT, whereas only 37% of afebrile patients had detectable levels of *Pf*HRPII. Fever was also associated, nonsignificantly with increased odds of positive results on all tested diagnostic mechanisms in univariate analysis, and a statistically significant relationship with odds of positive BUS-RDT result. However, the relationship did not maintain statistical significance when controlling for protein content

and parasitemia, and there is no correlation between fever and overall protein content itself in this data. Regardless, there is an important trend towards higher odds of protein detection in febrile patients. Though this can be seen as a possible problem in terms of finding all potential reservoirs of parasites, a diagnostic that is only reliable when used on febrile patients will still help prevent incorrect prescription of antimalarials to febrile patients presenting at local health centers. There was also an interesting trend in the odds of positive results on all tests, which increased significantly (by two fold or more) with each increase in categorical parasitemia level. This also adds to the possibility that any urine-based test would be most useful only on symptomatic patients with high parasitemia, and implies that urinary *Pf*HRPII may be a strong indicator of overall parasitemia as well.

Analysis was also performed to discern whether this 100-fold dilution was likely to be the same for all proteins, or if there was *Pf*HRPII-specific concentration manifesting itself. Given that normal plasma contains about 80 mg/mL of protein, and the geometric mean concentration of *Pf*HRPII in plasma was approximately 1500 ng/mL by ImmunoPCR for microscopy-positive patients, so *Pf*HRPII is about 1/53,333 in plasma. In the urine, total protein content had a geomean of 122 micrograms/mL in microscopy-positive patients, while *Pf*HRPII content was about 10.5 ng/mL. Thus, in urine, *Pf*HRPII is about 1/12,000 of the protein content. This may be due to its relatively hardy nature, such that other blood proteins degrade.

Another issue with urine-based diagnostics are the presence of blood in urine, and whether that is a factor for being able to detect *Pf*HRPII. There was otherwise no pattern of increased proportion of patients with bloody urine who received positive or negative diagnoses, implying that this is unlikely to be the only explanation for being able to detect *Pf*HRPII in urine.

In summary, although *Pf*HRPII is detectable in urine via multiple techniques and may be particularly promising as a biomarker in symptomatic patients, technology will require at least a 100 fold increase in sensitivity or testable sample volume to make urinary diagnosis of malaria a viable option. Future analyses could include continued exploration of the nanoparticle technology using greater volumes of urine and potentially testing for higher-affinity particles, as well as continuing to explore other correlates of *Pf*HRPII detection, including fever and level of parasitemia.

Works Cited

1. WHO. Malaria fact sheet. http://www.who.int/mediacentre/factsheets/fs094/en/. Accessed August 29, 2016.
2. Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI. The global distribution of clinical episodes of Plasmodium falciparum. *Nature*. 2005;434(3):214. doi:10.1029/2002gb001991.
3. CDC. Elimination of malaria in the United States (1947-1951). http://www.cdc.gov/malaria/about/history/elimination_us.html. Accessed August 29, 2016.
4. Cullen KA, Arguin PM. Malaria surveillance--United States, 2012. *MMWR Surveill Summ*. 2014;63(12):1-22. doi:10.1016/S0887-7963(98)80014-X.
5. Hay S, Guerra C, Tatem A, Noor A, Snow R. The global distribution and population at risk of malaria: past, present, and future. *Lancet Infect Dis*. 2004;4(June). doi:10.1016/S1473-3099(04)01043-6.
6. MALWEST. *Plasmodium* life cycle. http://www.malwest.gr/en-us/malaria/informationforhealthcareprofessionals/plasmodiumlifecycle.aspx. Accessed August 29, 2016.
7. CDC. CDC - Malaria - About Malaria - Biology - Malaria Parasites. USA Government. http://www.cdc.gov/malaria/about/biology/parasites.html. Published 2012.
8. Bhattarai A, Ali AS, Kachur SP, et al. Impact of artemisinin-based combination therapy and insecticide-treated nets on malaria burden in Zanzibar. *PLoS Med*. 2007;4(11):1784-1790. doi:10.1371/journal.pmed.0040309.
9. CDC. Insecticide-treated Bed Nets. Malaria.
10. Rehman AM, Coleman M, Schwabe C, et al. How much does malaria vector control quality matter: The epidemiological impact of holed nets and inadequate

indoor residual spraying. *PLoS One*. 2011;6(4). doi:10.1371/journal.pone.0019205.

11. Bhatt S, Weiss DJ, Cameron E, et al. The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015. *Nature*. 2015;526(7572):207-211. doi:10.1038/nature15535.
12. Kleinschmeidt I, Schwabe C, Shiva M, et al. Combining indoor residual spraying and insecticide-treated nets interventions. *Am J Trop Med Hyg*. 2009;81(3):519-524.
13. Hsiang MS, Hwang J, Tao AR, et al. Mass drug administration for the control and elimination of *Plasmodium vivax* malaria: an ecological study from Jiangsu province, China. *Malar J*. 2013;12(1):1-14. doi:10.1186/1475-2875-12-383.
14. WHO Evidence Review Group. Mass drug administration, mass screening and treatment and focal screening and treatment for malaria. 2015;(September):1-27. http://www.who.int/malaria/mpac/mpac-sept2015-erg-mda-report.pdf.
15. Newby G, Hwang J, Koita K, et al. Review of mass drug administration for malaria and its operational challenges. *Am J Trop Med Hyg*. 2015;93(1):125-134. doi:10.4269/ajtmh.14-0254.
16. Griffin JT, Hollingsworth TD, Okell LC, et al. Reducing *Plasmodium falciparum* malaria transmission in Africa: A model-based evaluation of intervention strategies. *PLoS Med*. 2010;7(8). doi:10.1371/journal.pmed.1000324.
17. Steffen R, Fuchs E, Schildknecht J, et al. Mefloquine compared with other malaria chemoprophylactic regimens in tourists visiting East Africa. *Lancet*. 1993;341(8856):1299-1303. doi:10.1016/0140-6736(93)90814-W.
18. Ferri F. Malaria. In: *Ferri's Color Atlas and Text of Clinical Medicine*. Elsevier Inc.; :1159.
19. MacKintosh CL, Beeson JG, Marsh K. Clinical features and pathogenesis of

severe malaria. *Trends Parasitol*. 2004;20(12):597-603. doi:10.1016/j.pt.2004.09.006.

20. English M, Punt J, Mwangi I, McHugh K, Marsh K. Clinical overlap between malaria and severe pneumonia in African children in hospital. *Trans R Soc Trop Med Hyg*. 1996;90(6):658-662. doi:10.1016/S0035-9203(96)90423-X.
21. Ukwaja KN, Aina OB, Talabi AA. Clinical overlap between malaria and pneumonia: Can malaria rapid diagnostic test play a role? *J Infect Dev Ctries*. 2011;5(3):199-203. doi:10.3855/jidc.945.
22. Sherman IW, Eda S, Winograd E. Cytoadherence and sequestration in *Plasmodium falciparum*: Defining the ties that bind. *Microbes Infect*. 2003;5(10):897-909. doi:10.1016/S1286-4579(03)00162-X.
23. Idro R, Marsh K, John CC, Newton CRJ. Europe PMC Funders Group Cerebral Malaria ; Mechanisms Of Brain Injury And Strategies For Improved Neuro-Cognitive Outcome. 2011;68(4):267-274. doi:10.1203/PDR.0b013e3181eee738.Cerebral.
24. UNICEF. Fact sheet: Malaria, a global crisis. http://www.unicef.org/media/media_20475.html.
25. Watsierah CA, Ouma C. Access to artemisinin-based combination therapy (ACT) and quinine in malaria holoendemic regions of western Kenya. *Malar J*. 2014;13:290. doi:10.1186/1475-2875-13-290.
26. Wiseman V, Kim M, Mutabingwa TK, Whitty CJM. Cost-effectiveness study of three antimalarial drug combinations in Tanzania. *PLoS Med*. 2006;3(10):1844-1850. doi:10.1371/journal.pmed.0030373.
27. Luxemburger C, Nosten F, Kyle DE, Kiricharoen L, Chongsuphajaisiddhi T, White NJ. Clinical features cannot predict a diagnosis of malaria or differentiate the infecting species in children living in an area of low transmission. *Trans R Soc*

Trop Med Hyg. 1998;92(1):45-49. doi:10.1016/S0035-9203(98)90950-6.

28. Mazigo HD, Meza W, Ambrose EE, Kidenya BR, Kweka EJ. Confirmed malaria cases among children under five with fever and history of fever in rural western Tanzania. *BMC Res Notes*. 2011;4:359. doi:10.1186/1756-0500-4-359.
29. Mbonye AK, Lal S, Cundill B, Hansen KS, Clarke S, Magnussen P. Treatment of fevers prior to introducing rapid diagnostic tests for malaria in registered drug shops in Uganda. *Malar J*. 2013;12(1):131. doi:10.1186/1475-2875-12-131.
30. Cohen J, Dupas P, Schaner S. Price subsidies, diagnostic tests, and targeting of malaria treatment: Evidence from a randomized controlled trial. *Am Econ Rev*. 2015;105(2):609-645. doi:10.1257/aer.20130267.
31. Arrow KJ, Panosian C, Gelband H. *Saving Lives, Buying Time: Economics of Malaria Drugs in an Age of Resistance*.; 2004. doi:ISBN-10: 0-309-09218-3.
32. UNICEF. Malaria mortality among children under five is concentrated in sub-Saharan Africa. http://data.unicef.org/child-health/malaria.html#sthash.mjlk11Ir.dpuf. Published 2016. Accessed August 29, 2016.
33. Moody A. Rapid diagnostic tests for malaria parasites. *Clin Microbiol Rev*. 2002;15(1):66-78. doi:10.1128/CMR.15.1.66-78.2002.
34. Milne LM, Kyi MS, Chiodini PL, Warhurst DC. Accuracy of routine laboratory diagnosis of malaria in the United Kingdom. *J Clin Pathol*. 1994;47(8):740-742. doi:10.1136/jcp.47.8.740.
35. Harchut K, Standley C, Dobson A, et al. Over-diagnosis of malaria by microscopy in the Kilombero Valley, Southern Tanzania: an evaluation of the utility and cost-effectiveness of rapid diagnostic tests. *Malar J*. 2013;12(1):159. doi:10.1186/1475-2875-12-159.
36. Coleman RE, Maneechai N, Rachaphaew N, et al. Comparison of field and expert

laboratory microscopy for active surveillance for asymptomatic *Plasmodium falciparum* and *Plasmodium vivax* in Western Thailand. *Am J Trop Med Hyg*. 2002;67(2 SUPPL.):141-144.

37. WHO. Malaria Rapid Diagnostic Test Performance. 2011;3.

38. Miller E, Sikes HD. Diagnostic Tests in Global Health. 2015. doi:10.5772/61114.Addressing.

39. Bousema T, Okell L, Felger I, Drakeley C. Asymptomatic malaria infections: detectability, transmissibility and public health relevance. *Nat Rev Microbiol*. 2014;12(12):833-840. doi:10.1038/nrmicro3364.

40. Murphy SC, Prentice JL, Williamson K, et al. Real-time quantitative reverse transcription PCR for monitoring of blood-stage *Plasmodium falciparum* infections in malaria human challenge trials. *Am J Trop Med Hyg*. 2012;86(3):383-394. doi:10.4269/ajtmh.2012.10-0658.

41. Slater HC, Ross A, Ouedraogo AL, et al. Assessing the impact of next-generation rapid diagnostic tests on *Plasmodium falciparum* malaria elimination strategies. *Nature*. 2015;528(7580):S94-S101. http://dx.doi.org/10.1038/nature16040.

42. Kifude CM, Rajasekariah HG, Sullivan DJ, et al. Enzyme-linked immunosorbent assay for detection of *Plasmodium falciparum* histidine-rich protein 2 in blood, plasma, and serum. *Clin Vaccine Immunol*. 2008;15(6):1012-1018. doi:10.1128/CVI.00385-07.

43. Cellabs. Malaria antigen CELISA.

44. Mayor A, Moro L, Aguilar R, et al. How hidden can malaria be in pregnant women? diagnosis by microscopy, placental histology, polymerase chain reaction and detection of histidine-rich protein 2 in plasma. *Clin Infect Dis*. 2012;54(11):1561-1568. doi:10.1093/cid/cis236.

45. Noedl H, Yingyuen K, Laoboonchai A, Fukuda M, Sirichaisinthop J, Miller RS.

Sensitivity and specificity of an antigen detection ELISA for malaria diagnosis. *Am J Trop Med Hyg*. 2006;75(6):1205-1208.

46. Bashir IM, Otsyula N, Awinda G, Spring M, Schneider P, Waitumbi JN. Comparison of PfHRP-2/pLDH ELISA, qPCR and Microscopy for the Detection of *Plasmodium* Events and Prediction of Sick Visits during a Malaria Vaccine Study. *PLoS One*. 2013;8(3). doi:10.1371/journal.pone.0056828.
47. Diggle E, Asgary R, Gore-Langton G, et al. Perceptions of malaria and acceptance of rapid diagnostic tests and related treatment practises among community members and health care providers in Greater Garissa, North Eastern Province, Kenya. *Malar J*. 2014;13:1-12. doi:10.1186/1475-2875-13-502.
48. Rodriguez-del Valle M, Quakyi IA, Amuesi J, Quaye JT, Nkrumah FK, Taylor DW. Detection of antigens and antibodies in the urine of humans with *Plasmodium falciparum* malaria. *J Clin Microbiol*. 1991;29(6):1236-1242.
49. Genton B, Paget S, Beck H, Gibson N, Alpers M, Hii J. Diagnosis of *Plasmodium falciparum* infection using ParaSight(R)-F test in blood and urine of Papua New Guinean children. *Southeast Asian J Trop Med Public Heal*. 1998;29(1):35-40.
50. Wyngaarden JB, Smith Jr. LH, Bennett JC. *Cecil Textbook of Medicine*. 19th ed. Philadelphia, PA: W. B. Saunders; 1992.
51. Oguonu T, Shu E, Ezeonwu BU, et al. The performance evaluation of a urine malaria test (UMT) kit for the diagnosis of malaria in individuals with fever in south-east Nigeria: cross-sectional analytical study. *Malar J*. 2014;13:403. doi:10.1186/1475-2875-13-403.
52. WHO. Selecting and procuring RDTs for malaria. http://www.who.int/malaria/areas/diagnosis/rapid-diagnostic-tests/selection-procurement/en. Published 2015. Accessed August 29, 2016.
53. Howard RJ, Uni S, Aikawa M, et al. Secretion of a malarial histidine-rich protein

(Pf HRP II) from *Plasmodium falciparum*-infected erythrocytes. *J Cell Biol*. 1986;103(4):1269-1277. doi:10.1083/jcb.103.4.1269.

54. Desakorn V, Dondorp AM, Silamut K, et al. Stage-dependent production and release of histidine-rich protein 2 by *Plasmodium falciparum*. *Trans R Soc Trop Med Hyg*. 2005;99(7):517-524. doi:10.1016/j.trstmh.2004.11.014.

55. Dondorp AM, Desakorn V, Pongtavornpinyo W, et al. Estimation of the total parasite biomass in acute falciparum malaria from plasma PfHRP2. *PLoS Med*. 2005;2(8):0788-0797. doi:10.1371/journal.pmed.0020204.

56. Iqbal J, Siddique A, Jameel M, Hira PR. Persistent histidine-rich protein 2, parasite lactate dehydrogenase, and panmalarial antigen reactivity after clearance of *Plasmodium falciparum* monoinfection. *J Clin Microbiol*. 2004;42(9):4237-4241. doi:10.1128/JCM.42.9.4237-4241.2004.

57. Pava Z, Echeverry DF, Díaz G, Murillo C. Short report: Large variation in detection of histidine-rich protein 2 in *Plasmodium falciparum* isolates from Colombia. *Am J Trop Med Hyg*. 2010;83(4):834-837. doi:10.4269/ajtmh.2010.10-0075.

58. Rubach MP, Mukemba J, Florence S, et al. Plasma *Plasmodium falciparum* histidine-rich protein-2 concentrations are associated with malaria severity and mortality in Tanzanian children. *PLoS One*. 2012;7(5):3-7. doi:10.1371/journal.pone.0035985.

59. Mikita K, Thakur K, Anstey NM, et al. Quantification of *Plasmodium falciparum* Histidine-rich protein-2 in cerebrospinal spinal fluid from cerebral malaria patients. *Am J Trop Med Hyg*. 2014;91(3):486-492. doi:10.4269/ajtmh.14-0210.

60. Luchini A, Geho DH, Bishops B, et al. Smart hydrogel particles: Biomarker harvesting: One-step affinity purification, size exclusion, and protection against degradation. *Nano Lett*. 2008;8(1):350-361. doi:10.1021/nl072174l.

61. Tamburro D, Fredolini C, Espina V, et al. Multifunctional core-shell nanoparticles: Discovery of previously invisible biomarkers. *J Am Chem Soc*. 2011;133(47):19178-19188. doi:10.1021/ja207515j.

62. Luchini A, Espina V, Liotta L a. Protein painting reveals solvent-excluded drug targets hidden within native protein-protein interfaces. *Nat Commun*. 2014;5:4413. doi:10.1038/ncomms5413.

63. Fredolini C, Tamburro D, Gambara G, et al. Nanoparticle technology: Amplifying the effective sensitivity of biomarker detection to create a urine test for hGH. In: *Drug Testing and Analysis*. Vol 1. ; 2009:447-454. doi:10.1002/dta.96.

64. Longo C, Patanarut A, George T, et al. Core-shell hydrogel particles harvest, concentrate and preserve labile low abundance biomarkers. *PLoS One*. 2009;4(3). doi:10.1371/journal.pone.0004763.

65. Magni R, Espina BH, Shah K, et al. Application of Nanotrap technology for high sensitivity measurement of urinary outer surface protein A carboxyl-terminus domain in early stage Lyme borreliosis. *J Transl Med*. 2015;13(1):346. doi:10.1186/s12967-015-0701-z.

66. Castro-Sesquen YE, Gilman RH, Galdos-Cardenas G, et al. Use of a Novel Chagas Urine Nanoparticle Test (Chunap) for Diagnosis of Congenital Chagas Disease. *PLoS Negl Trop Dis*. 2014;8(10). doi:10.1371/journal.pntd.0003211.

67. Ehrich JH, Horstmann RD. Origin of proteinuria in human malaria. *Trop Med Parasitol*. 1985;36(1):39-42.

68. Carroll MF. Proteinuria in Adults: a diagnostic approach. *Am Fam Physician*. 2000;62(6).

69. Nand N, Aggarwal H, Sharma M, Singh M. Systemic Manifestations of Malaria. *J Indian Acad Clin Med*. 2001;2(3):189-194.

70. Ephraim RKD, Tashie W, Agbodzakey H, et al. Dipstick urinalysis findings in

children with *Plasmodium falciparum* in the South Tongu District: A case-control study. *Niger Med J*. 2015;56(4):292-296.

Curriculum Vitae

Nicole Assumpcao

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Education

Johns Hopkins School of Public Health
ScM Student – Molecular Microbiology and Immunology August 2014-September 2016
GPA: 3.92

Courses include: Principles of Immunology, Virology, Biology of Parasitism, Introduction to International Health, Statistical Methods in Public Health (4 terms), Epidemiologic Methods (3 terms), Project Development for Primary Health Care in Developing Countries, Implementation and Sustainability of Community-based Health Programs, Environment and Health in Low and Middle Income Countries, Managing Non-Governmental Organizations in the Health Sector, Large-Scale Effectiveness Evaluations, and others.

Santa Clara University
Bachelor of Science - Biology and Psychobiology, Summa cum Laude Sept. 2013
GPA: 3.95
Courses included: Anatomy, Physiology, Cellular and Molecular Biology, Medical Microbiology, Neurobiology, Neuropsychology

Awards and Honors:

Phi Beta Kappa Honors Society (2013), Psi Chi Honors Society (2011), Dean's list 2010-2013; Susan Valeriote and Kenneth A. Goldman Family Endowed Scholarship (2013)

Lakeside School – High School Diploma
June 2009

Languages Spoken: English**,** Portuguese (fluent), Spanish (working proficiency), Chinese (basic)

Research Experience
ScM Thesis Research January-September 2016

- Under the guidance of Dr. David Sullivan and Dr. Robert Gilman, performed tests to look for and quantify HRP2 protein in the urine of malaria-infected patients.
- Worked collaboratively with Dr. Lance Liotta and Dr. Alessandra Luchini at George mason University to find a hydrogel nanoparticle to aid in concentration of HRP2 from urine samples with the hope of developing a more sensitive urine-based diagnostic test.

Global Health Established Field Placement Award June 2015-August 2015

- Received award through Center for Global Health at Johns Hopkins to perform research at a field site in Iquitos, Peru.
- Performed community surveys on water usage, utilizing cultural competency to dialogue with community members and work collaboratively with fieldworkers.

- Performed basic scientific tests for water quality, to be matched up with survey data for epidemiologic study on risk exposure and diarrheal disease in a five-year cohort study of children in a periurban environment in the Amazon.

Student Rotations **January 2015-June 2015**

- Performed PCR analysis on urine samples for detection of *Strongyloides stercoralis* DNA.
- Generated cDNA and ssRNA for gene knockouts in mosquitoes, then created nanoparticles for feeding/injection.
- Reared, manipulated, and injected mosquitoes to manipulate expression of genes relating to transmission of vector-borne diseases.

Research Assistant – Santa Clara Biology Department **June 2013-December 2013**

- Worked in the lab of Dr. Teresa Ruscetti and Dr. Christelle Sabatier examining the connection of microbiological organisms colonizing the gut of *c. elegans* and their behavior and neurobiology, leading research on the naturally colonizing bacteria in wild *c. elegans* and transit time of bacteria through *c. elegans* gut.
    - Developed an assay for collection of wild nematodes from soil using bacteria and chemoattractants.
    - Developed an assay to determine the bacterial content of the gut of *c. elegans*.
    - Practiced aseptic technique in husbandry of bacterial strains (including anaerobic bacteria).
    - Trained fellow research assistants in aseptic technique in plate pouring and formulation of reagents.

Research Assistant – Efficient Learning and Memory Lab **March 2011-August 2012**

- Ran participants and compiled data under Dr. Patricia Simone and Dr. Matthew Bell, researching the effect of massed and spaced learning paradigms on long-term retention across ages.
- Maintained data spreadsheets and utilized Excel to perform basic data analysis.

Presentations

World Malaria Day – Johns Hopkins University **Spring 2016**

- Presented poster on preliminary findings regarding nanoparticle-mediated concentration of proteins in urine of malaria-infected patients

Northern California American Society for Microbiology Meeting **Fall 2013**

- Presented poster on numerous research methods utilized to determine transit time of bacterial foods through *c. elegans* gut.

Western Psychological Association Convention **Spring 2012**

- Presented in a symposium regarding theory of “massed” versus “spaced” learning paradigms and their effect on long-term memory retention with Efficient Learning and Memory Lab.

California Cognitive Science Conference **Spring 2011**

- Held at the University of California at Berkeley. Poster presentation of data on massed and spaced learning with Efficient Learning and Memory Lab.

Western Psychology Conference for Undergraduate Research **Spring 2011**

- Held at Saint Mary's College. Poster presentation of data on massed and spaced learning.

Teaching Experience

Teaching Assistant - Human Physiology **Fall 2015, Summer 2016**

- Helped students at the Johns Hopkins School of Nursing to learn and retain information pertinent to human physiology. This included answering student questions as well as grading discussion posts on a weekly basis.

Peer Educator – Life Writing **Spring 2013**

- Helped facilitate autobiographical writing in Life Writing course taught by Juan Velasco, including discussing difficult life topics in one-on-one sessions with students.
- Edited student work for clarity, grammatical errors, and flow.

Teaching Assistant - Human Neuropsychology **Fall 2011**

- Compiled comprehensive reviews of challenging material for undergraduate students in a Neuropsychology course taught by Dr. Patricia Simone, simplifying complex concepts to make them more accessible to students.

Volunteer Experience

English Teacher – Casa de la Solidaridad **August 2012-December 2012**

- Taught English to children in a marginalized community outside San Salvador, El Salvador while studying material pertaining to issues of social justice in the community.
- Maintained a friendly demeanor even in challenging situations and built strong relationships with Salvadoran community.